PhD Project Report

Project IV.4, meta-phos:

" Phosphorus as a metabolic regulator during environmental stress"

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Start of work:	13th August, 2020
End of work:	31st October, 2023

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1. CONCEPT/INTRODUCTION

Background

Energy is a basic necessity for the survival of living organisms such that biological, physiological and chemical processes in cells strictly rely on its constant supply. This energy is mostly stored as adenosine triphosphate (ATP) molecules in cells and is generated by several metabolic pathways. In animals, food consumed is the major source of energy and converted into ATP through processes like glycolysis, and mitochondrial electron transport and oxidative phosphorylation. After digestion, sugars and glucose through the glycolytic pathways and glycolysis are converted to pyruvate in the cytosol which is then transported to the mitochondria for acetyl CoA production. Also, the food particles can be metabolized directly for acetyl CoA production in the mitochondria. The acetyl CoA is then transferred into the tricarboxylic acid (TCA) cycle where reactions produce NADH and FADH which transport electrons into the electron transport chain. This electron flow also pumps protons through the F_1F_0–ATP synthase into the mitochondrial intermembrane space consuming large quantities of oxygen and generating ATP through several processes with the most pronounced being phosphorylation of ADP (Watt et al. 2010).

It is obvious from the above that majority of the generation of ATP occurs in the mitochondria with oxygen as the terminal electron acceptor through a process of oxidative phosphorylation. The oxidative phosphorylation process involves the flow of electrons through several mitochondrial respiratory enzyme complexes (complex I, III, IV, V). The path of the flow starts with NADH → NADH dehydrogenase complex → ubiquinone → cytochrome *b*-c_1 complex → cytochrome *c* → cytochrome oxidase complex → molecular oxygen (O_2) (Alberts B, Johnson A, Lewis J 2002).

Mitochondria aside being the powerhouse of the cell have been reported to coordinate cellular adaptation to stressors such as nutrient deprivation, oxidative stress, DNA damage and endoplasmic. reticulum (ER) stress. Most of these stresses are generated from changing environmental conditions like oxygen deficiency, temperature among others.

State of the Art

Hypoxia is used to characterize conditions of oxygen deficiency. Oxygen is key to the survival of aerobic organisms due to its involvement in mitochondrial oxidative phosphorylation (OXPHOS) for the generation of ATP. Environmentally, aquatic organisms are more prone to these conditions whiles most terrestrial organisms experience oxygen deficiency only at high altitudes where there is decreased partial pressure of oxygen (PO2) (Richards 2011). In shallow, deep, and benthic habitats, hypoxia occurs due to water stratification in the summer and ice formation during the winter which prevents water mixing and air

exchange (Fukushima et al. 2017; Breitburg et al. 2018). Also, warming of surface waters and pollution due to high available nutrient which drives bacterial respiration depletes oxygen and creates dead zones making available oxygen for aerobic organisms limited. However, at the cellular level, both terrestrial and aquatic organisms are prone to conditions of hypoxia. Terrestrial organisms experience hypoxic condition during ischemia, impaired oxygen diffusion in the lungs, impaired cardio-vascular function, or hypoventilation (Burtscher et al. 2018).

Mammalian and non-mammalian studies have revealed that hypoxia surge the rate of reactive oxygen species (ROS) production. Hypoxia renders the cell in a reductive state causing an increase in reducing equivalents (mostly NADH and $FADH_2$) especially in the mitochondria. It also makes electrons readily available for reduction reactions such as the reduction of O_2 to superoxide (Reactive Oxygen Species, ROS) (Clanton 2007). Superoxides through other reactions are converted into ROS like hydrogen peroxide (H_2O_2). H_2O_2 is relatively stable and due to membrane permeability, it can diffuse into the cytosol where it can be eliminated by the antioxidant system and through other means. Excess production of H_2O_2 above the cells antioxidant system leads to its interaction with the metal-catalyzed Fenton reaction to produce a much lethal ROS, the hydroxyl radical (HO^-). HO^- causes oxidative damage and stress and can eventually lead to cell death. According to Dawson et al. (Dawson et al. 1993), hypoxia- or ischemia-induced ROS production may require conditions of low-flow or intermittent ischemia where cycles of anoxia and reoxygenation are occurring almost concurrently leading to a state of redox disequilibrium and may be the most common form of hypoxia in living organisms (Clanton and Klawitter 2001). Although stressed/hypoxic cells have been observed to produce ROS, healthy cells also produce ROS. Generally, mitochondria ROS (superoxide) is produced in all the OXPHOS complexes with Complex 1 being the main source in healthy cells.

Studies conducted on hypoxia/reoxygenation (H/R) or ischemia/reperfusion (I/R) stress has reported that major damage to tissues mostly occurs when the limiting substrate, oxygen is reintroduced (reoxygenation/reperfusion) and not when it is limiting (hypoxia/ischemia). This is because upon reoxygenation, there is an increase in the production of mitochondrial ROS that may oxidize proteins, lipids and DNA (Kowaltowski et al. 2009). For instance, during ischemia, succinate accumulates in the heart. Upon reperfusion succinate acts as an electron source which drives reverse electron transfer (RET), where electrons travel from succinate to the ubiquinone pool via complex II. They move further on to complex I in reverse, using the elevated proton motive force to generate ROS (Murphy 2009; Chouchani et al. 2016). Reoxygenation/reperfusion in some hypoxic sensitive species like the bay scallop and some terrestrial mammals also resulted in the collapse of the electron transport system (ETS) capacity and

mitochondrial membrane potential whiles hypoxic tolerant species like oysters, fish and turtles had the ETS capacity rapidly restored or enhanced during reoxygenation (Kadenbach et al. 2011; Sokolov et al. 2019; Ivanina et al. 2016; Pamenter et al. 2016). Despite the conservation ability of ETS capacity in the tolerant species, OXPHOS rates was generally reduced in both sensitive and tolerant species with the tolerant species able to reverse back to normal (Onukwufor et al. 2016). Hence, inhibition of OXPHOS rate may be a general response to hypoxia.

Mammals are known for their sensitivity to hypoxia or ischemia/reperfusion stress. Several mammalian models (mouse, rats, pigs) have been explored to find solutions to some pathological condition in humans (Baehr, Klymiuk, and Kupatt 2019; L. G. M. Wijermars et al. 2016). Conditions such as stroke, cardiac arrest, organ transplantation failures among other are mostly preceded by ischemia and upon reperfusion, tissue damage occurs which may sometimes lead to death (Leonie G.M. Wijermars et al. 2016). These injuries have been associated with ROS (Horstkotte et al. 2011), imbalance of Ca^{2+} homeostasis (Luongo et al. 2017), mitochondrial damage (Baines et al. 2005) and, consequently, cell death. Susceptibility to these injuries have also been observed to differ in different tissue with the mammalian brain consented to be the most sensitive compared to the skeletal muscle, liver, etc. With the vast studies, mammalian models have become a benchmark for most studies relating to hypoxia and oxygenation stress.

In marine organisms, hypoxia is a key stressor hence some organisms like mollusks have developed adaptive mechanism to hypoxia tolerance. Bivalves like clams, oysters and mussels have been reported to be much tolerant than others (scallops), fishes and crustaceans. The hypoxia tolerant bivalves have developed metabolic mechanisms to aid in their adaptation such as metabolic rate depression, reducing toxic end products accumulation and energy reserves (Sokolova I, Bock C, and Pörtner 2000). However, reoxygenation after hypoxic stress has been observed to cause oxidative stress and damage even in the tolerant species due to an increase in ROS from the mitochondrial ETS (Andrienko et al. 2017). Various research has sort to find remedies and ways to curb this damage by including conditions known to affect mitochondrial ROS generation as positive and negative controls (e.g. OXPHOS inhibitors, mitochondrial uncoupling, and O_2 depletion). Results from hypoxia-tolerant animals like the intertidal mollusks, crucian carp, and fish have revealed some mechanism adopted to deal with oxidative stress generated as a result of H/R. Some of these mechanisms includes heightened antioxidant activities, increase in mitochondrial quality control mechanisms such as proteases and HSPs, and inhibition of pathways channeling electrons to ubiquinone (Sokolov et al. 2019; Inna Sokolova 2018; Inna M. Sokolova, Sokolov, and Haider 2019).

Although much research has been conducted in this field, there is a lot more to discover especially with respect to the mitochondrial mechanisms of response to hypoxia.

Mitochondria are known to be capable of utilizing diverse metabolic substrates (including carbohydrates, fatty acids, and amino acids) to generate ATP. The nature of the respiratory substrate affects the stoichiometry of oxygen consumption to ATP production (Leverve and Fontaine 2001) and ROS generation rates (Quinlan et al. 2013) hence some substrates might be preferred than others under stressful conditions. For example, Drosophila showed preference for succinate and proline than NADH-linked substrates (pyruvate, glutamate) under high temperature conditions. In the hypoxia-tolerant goldfish, the brain mitochondria switch from carbohydrate to fatty acid oxidation during hypoxia (Farhat et al. 2021). These suggests that different substrate oxidation especially under conditions of stress affect mitochondrial mechanisms as some substrate might enhance tolerance than others. Unfortunately, there are limited studies on the metabolic flexibility in mitochondrial substrate utilization under H/R stress. These investigations are crucial as they might have major implications for bioenergetics and redox balance, hence warranting further investigation.

Project summary

This project explored mitochondrial and molecular mechanisms regulating oxidative phosphorylation and oxidative stress during hypoxia-reoxygenation stress in marine bivalves (oysters, scallops) and mammals. Mitochondria from various tissues and animals are different in terms of sensitivity hence this project investigated tissues with differing sensitivities to H/R (brain and muscle in pigs, gills, and digestive gland in bivalves) and hypoxia tolerant animals (oysters) and hypoxia sensitive animals (pigs and scallops). Additionally, due to the variations in coastal hypoxic episodes (lasting from several hours (during diurnal cycles of photosynthesis and respiration) to days and weeks in coastal dead zones (Vaquer-Sunyer and Duarte, 2008)), mitochondrial responses in marine bivalves were investigated under differing hypoxic periods (acute and chronic hypoxia). These results will help to evaluate and propose a benchmark between mammals and bivalves with respect to mitochondrial mechanisms regulated during H/R stress and further understand mechanisms regulating hypoxia tolerance and sensitivities.

2. MATERIAL AND METHODS

Chemicals

Chemicals for the experiments were purchased from Sigma Aldrich (Munich, Germany), Fisher Scientific (Schwerte, Germany), or Carl Roth (Karlsruhe, Germany).

Animals

The experimental animals were Pigs, oysters, and scallops. The tissues of focus were the skeletal muscles and brain in the pigs and gills and digestive gland in the scallops and oysters.

Methods

The mitochondria response of different tissue and animals to hypoxia/reoxygenation stress were evaluated together with its reactive oxygen species production and scavenging. All mitochondria experiments were conducted in the Oroborus. Oxygen consumption rate (MO2) and hydrogen peroxide (H_2O_2) efflux rate were measured in isolated mitochondria at 15±1°C using a high resolution Oxygraph 2-k respirometer (Oroboros, Innsbruck, Austria) with integrated DatLab 6 software for bivalves and at 37°C in isolated brain and skeletal muscle cells. All hypoxia exposure were conducted *in vitro* in the Oroborus. Details of experimental methods are outlined in (Sokolov, Adzigbli, Markert, et al. 2021; Adzigbli, Sokolov, Wimmers, et al. 2022). Method summaries are presented below.

Pig experiment

- Preparation of cell isolates
- Cell permeabilization
- SUIT - Oxygen consumption and ROS efflux measurement under normoxia and acute hypoxia (15 min hypoxia, 10 min reoxygenation)
- Protein content measurement
- Sample collection for transcript analysis
- Sample preparation for LC-MS/MS analysis: Total cell proteins and phosphoproteins
- RNA extraction
- Transcript measurement

Oyster experiment

- Mitochondria isolation
- SUIT - Oxygen consumption and ROS efflux measurement under acute hypoxia (15 min hypoxia, 10 min reoxygenation), chronic cyclic hypoxia (5 cycles of 15 min hypoxia, 10 min reoxygenation),

and chronic long-term hypoxia (90 min hypoxia, 10 min reoxygenation). SUIT measurements were conducted using 3 main metabolic substrate fuels: pyruvate, palmitate and succinate.

- Protein content measurement
- Measurement of oxidative stress indices using ELISA

Scallop experiment

- Mitochondria isolation
- SUIT - Oxygen consumption and ROS efflux measurement under acute hypoxia (15 min hypoxia, 10 min reoxygenation), and chronic long-term hypoxia (90 min hypoxia, 10 min reoxygenation). SUIT measurements were conducted using 3 main metabolic substrate fuels: pyruvate, palmitate and succinate.
- Protein content measurement
- Measurement of oxidative stress indices using ELISA
- Metabolite analysis of tissues subjected to in vivo H/R stress (22-hour hypoxia, 1.5-hour reoxygenation or 24-hour reoxygenation)

Comparative transcriptomic experiment

- In vivo hypoxia exposure in oysters, mussels, and scallops
- Tissue excision and RNA extraction
- Transcript measurements

Data analysis

- Origin and Microsoft excel 2010 were used for tabulating results and drawing simple graphs.
- IBM SPSS Statistics v. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v. 7.02 (GraphPad Software Inc., La Jolla, CA, USA) software were used for statistical analysis.
- Meta-bo analyst was used for metabolite analysis.
- Principal component analysis (PCA) was used on the raw (non-transformed) metabolite data to reduce the dimensionality of the data set and compare the integrated biomarker profiles in different experimental groups.
- Data was presented as spreadsheet formats (.csv), image files (.TIF/JPEG) and free text documents (.txt).
- Data files were also published in online data repositories.

3. RESULTS/DISCUSSION SUMMARY

Pig experiment

Our experiment revealed both mitochondrial and molecular responses of porcine brain and muscle cells to hypoxia reoxygenation stress. Muscle cells were more tolerant (stable maximum ATP synthesis capacity) than the brain cells (decreased maximum ATP synthesis capacity). However, there was no difference in ROS efflux between both tissues. Additionally, COX activity decreased significantly after reoxygenation in both studied tissues. These response at the mitochondrial level were also supported by downstream transcript regulation. At the transcriptional level, analysed genes encoding COX subunits (COX6A1 in the muscle and COX6C and COX7A1 in the brain) were downregulated confirming the observation at the mitochondria levels.

Transcripts involved in HIF-1 regulation, apoptosis, redox homeostasis, glycolysis, ETS and TCA cycle, and antioxidant defence were identified from the study. Key Complex I subunits (NDUFA6, NDUFAB1, NDUFS1, NDUFAB1) were regulated in response to hypoxia reoxygenation stress. In the porcine muscle cells, an increase in the mitochondrial Complex I activity after reoxygenation was associated with a strong overexpression of NDUFS1 transcript. NDUFS1 is known to suppresses Complex I activity and increase ROS production in different mammalian cells. Therefore, its overexpression during hypoxia might thus represent an anticipatory response of the porcine muscle cells to support high Complex I activity and mitigate ROS during reoxygenation and might be responsible for the tolerance of the muscle cells. In the porcine brain cells, NDUFAB1 was suppressed and NDUFA6 overexpressed after reoxygenation. NDUFAB1 plays an important role in regulating ETS flux and ROS production in mitochondria and its overexpression mitigates ischemia–reperfusion injury in mammalian cells. Hence, its downregulation might be maladaptive during H/R and contribute to hypoxia sensitivity of the porcine brain cells.

The regulation of the key transcriptional regulator of hypoxia HIF-1 and its post-translationally regulator, prolyl hydroxylase domain protein (PHD) were observed after H/R stress in the porcine cells. HIF-1 upregulates the transcription of multiple glycolytic enzymes such as enolase 1, aldolase, hexokinase, and glyceraldehyde-3-phosphate dehydrogenase to enhance anaerobic ATP production under oxygen deficiency. Although HIF-1 was upregulated in this study, all glycolytic enzymes analysed were not affected by H/R stress in both tissues and this might be due to the short hypoxia exposure period. Also, antioxidant enzymes were also differentially regulated in both tissues after H/R stress. Taken together, these findings show that acute short-term H-R exposure did not induce strong oxidative stress consistent with the observation of the stable ROS emission during H-R stress in the isolated muscle and brain cells. For microRNA expressions, the porcine muscle cells suppressed 4 of the 34 studied miRNA in hypoxia and 9 suppressed in reoxygenation relative to the normoxic controls. In the porcine brain cells, substantially 17 of the 41 studied miRNAs were suppressed after H-R stress. This indicates that the

hypoxia-induced metabolic reorganization involving hypoxamiRs affects a larger swath of the cellular pathways in the porcine brain than in the muscle cells. (Adzigbli, Sokolov, Wimmers, et al. 2022).

Oyster experiment

In the Pacific oyster *Crassostrea gigas*, we observed that the two metabolically active tissues; gill and digestive gland have different utilization of mitochondria substrate fuels under normal oxygen conditions. The gill mitochondria generally showed higher OXPHOS respiration rate with glutamate, succinate as well as glutamate-and succinate-containing mixtures and the digestive gland mitochondria, with the mixture of glutamate, pyruvate, and succinate. Both gill and digestive gland tissues showed low preference for palmitate with the palmitate-driven ROS production 4-5-fold higher in the digestive gland mitochondria than the gill. Our findings suggests that the digestive gland tissue is metabolically less well adapted for amino acid and fatty acid oxidation compared to the gills.

Exposure to acute hypoxia further altered the substrate preference of the two tissues. The oxidation rate of Complex I (CI) substrates (NADH-linked) were suppressed after H/R stress in both tissues. However, the ROS efflux increased more than 100% in OXPHOS state but decreased in the LEAK state stipulating that the ROS generation during CI-dependent ETS activity was strongly dependent on the activity state of the mitochondria. Hence, the electron leak in the OXPHOS state was generally higher than that of the LEAK state after H/R stress. Overall, the opposing effects of H/R stress on ROS generation in the resting vs. actively phosphorylating oyster mitochondria make it difficult to predict physiological consequences of the H/R stress for oyster mitochondria respiring on CI substrates in vivo. Alternatively, the oxidation of a $FADH_2$-linked substrate (succinate) was enhanced after H/R stress in both oyster tissue mitochondria. The digestive gland mitochondria appeared particularly well adapted to succinate oxidation as succinate-driven proton leak was relatively low but enhanced OXPHOS activity after H/R stress opposed to that of the gill mitochondria where both proton leak and OXPHOS rates increased after H/R stress. Enhanced mitochondrial capacity for succinate oxidation might be adaptive during post-hypoxic recovery in oysters helping to rapidly restore the ATP levels and remove excess succinate. However, the elevated capacity for succinate oxidation induced by the H/R stress was associated with higher ROS production in oyster mitochondria but partially alleviated by rotenone. Furthermore, addition of pyruvate attenuated succinate-driven ROS efflux and electron leak in the H/R-stressed mitochondria suggesting low ROS production under the physiological conditions due to the presence of NADH-linked substrates such as pyruvate. (Sokolov, Adzigbli, Markert, et al. 2021)

Exposure to chronic cyclic hypoxia spiked a loss in mitochondrial respiratory capacity and the loss was substrate dependent; 55-73% for pyruvate oxidation, 71-73% for palmitate oxidation, and 51-52% for succinate oxidation. These declines were also reflected in the ROS efflux especially in the LEAK state of CI substrate oxidation however, ROS efflux or FEL rate in the succinate-energized oyster mitochondria was stable.

Exposure to chronic long-term hypoxia suppressed oyster respiratory capacity during both CI (pyruvate and palmitate) and CII (succinate) substrate oxidation. However, the suppression was doubled during CI substrates oxidation compared to the CII substrate. ROS efflux remained unchanged or slightly increased despite the suppressed oxidation rates of CI substrates leading to an increased fractional electron leak which also reflected in the oxidative damage marker (high accumulation of protein carbonyls). However, there was no increase in ROS efflux or FEL rate in the succinate-energized oyster mitochondria and no accumulation of the oxidative damage to proteins (indicated by protein carbonyls).

Taken together, palmitate oxidation generated the lowest respiratory flux under normoxia and the highest decline both under the different H/R exposure periods in oyster mitochondria. Additionally, CI substrates are not better suited for oysters under H/R stress due to the decline in respiratory capacity and elevated electron leak. However, succinate addition to the NADH-linked substrates can help alleviate the negative effects of H/R stress supporting the notion of positive metabolic effects of succinate in bivalve mitochondria. Succinate can therefore serve as a potential stress fuel in ectotherm mitochondria coupled with the high succinate oxidation capacity for mitochondrial stress tolerance.

Scallop experiment

Both gill and digestive gland mitochondria of the adult king scallop *Pecten maximus* had similar oxidation rate for all substrates except for pyruvate where the digestive gland had a lower oxidation rate than the gill. Despite similar respiratory capacity, ROS efflux was very different. The digestive gland generally had higher efflux and fractional electron leak with all substrates.

Exposure to acute H/R stress led to a decline in respiratory capacity of both gill and digestive gland mitochondria oxidizing both CI and CII substrates with palmitate recording the highest decline (71% in gill, 42% in digestive gland) and pyruvate the lowest. Despite the decline in respiratory capacity, ROS efflux and fractional electron leak was quite stable in the gill mitochondria but decline in the digestive gland mitochondria.

Exposure to chronic long-term H/R stress revealed similar decline in respiratory capacity like that experienced during acute H/R stress. However, succinate oxidation recorded the lowest decline (~27%) as opposed to pyruvate oxidation during acute H/R stress. Additionally, the decline in respiratory capacity of all substrate oxidation was also associated with a decline in ROS efflux and fractional electron leak in the digestive gland mitochondria.

Our findings suggest that pyruvate oxidation might be better suited under acute H/R stress and succinate oxidation under chronic long-term H/R stress in the scallop mitochondria. That notwithstanding, our experiment observed the sensitivity of scallops to H/R stress and this sensitivity was independent of exposure time and oxidative damage. Generally, the digestive gland mitochondria of the king scallop were more sensitive to H/R stress.

4. MILESTONES/PUBLICATIONS

No. SP	Description of milestone	Year
1	Intrinsic Mechanisms Underlying Hypoxia-Tolerant Mitochondrial Phenotype during Hypoxia-Reoxygenation Stress in a Marine Facultative Anaerobe, the Blue Mussel Mytilus edulis. Frontiers in Marine Science, 8:773734. 10.3389/fmars.2021.773734	2021
2	Tissue- and substrate-dependent mitochondrial responses to acute hypoxia-reoxygenation stress in a marine bivalve Crassostrea gigas (Thunberg, 1793). Journal of Experimental Biology, 225(1) 10.1242/jeb.243304	2021
3	Effects of hypoxia and reoxygenation on mitochondrial functions and transcriptional profiles of isolated brain and muscle porcine cells. Scientific Reports, 12, 19881. 10.1038/s41598-022-24386-0	2022
4	Mitochondrial responses to long-term and cyclic hypoxia depend on the oxidized fuel in a hypoxia-tolerant marine bivalve Crassostrea gigas	Submitted
5	Mitochondrial responses to acute and long-term hypoxia in a hypoxia-sensitive marine bivalve Pecten maximus	In writing

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