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Abschlussbericht

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Zuwendungsempfänger:	Prof. Inna Sokolova (Uni Rostock)
Kooperationspartner:	Prof. Klaus Wimmers (Leibniz FBN), Dr. Eugene Sokolova (Leibniz IOW), Dr. Siriluck Ponsuksili (Leibniz FBN)
Vorhabensbezeichnung:	MitoP: The role of reversible phosphorylation in regulation of mitochondrial bioenergetics
Laufzeit des Vorhabens:	01.03.2017-31.03.2018
Autoren:	Sokolova, Wimmers, Sokolov, Ponsuksili, Markert, Hinzke, Becher

Hinweis: Der Bericht sollte eine Länge von 8 bis 10 Seiten nicht überschreiten!

Inhaltsverzeichnis

* z.B. Beiträge auf Konferenzen, Publikationen (mit Status), Drittmittelanträge (mit Status)

1 Zusammenfassung und Schlussfolgerung

Mitochondria are key intracellular targets of hypoxia-reoxygenation (H/R) stress due to their central role in ATP production and reactive oxygen species (ROS) generation. Intertidal bivalves such as oysters *Crassostrea gigas* are adapted to frequent H/R cycles and maintain aerobic function despite frequent oxygen fluctuations. To gain insight into the molecular mechanisms of H/R tolerance, we assessed the shifts in mitochondrial (phospho)proteome and functional changes in *C. gigas* mitochondria during hypoxia and recovery. Oyster mitochondria maintained OXPHOS capacity despite a decline in cytochrome c oxidase activity during H/R stress. Rearrangements of the mitochondrial proteome involved upregulation of mitochondrial electron transport system and iron-binding proteins and suppression of the metabolic pathways that channel electrons to ubiquinone, possibly as a mechanism to limit ROS production during H/R stress. H/R stress led to upregulation of a mitophagic activator PGAM5 and dephosphorylation of metalloendopeptidase OMA1, indicating stimulation of mitochondrial quality control mechanisms. Changes in abundance and phosphorylation levels of key proteins involved in the mitochondrial protein homeostasis indicate suppression of the protein synthesis during hypoxia, likely as an energy-saving mechanism. Thus, shifts in the mitochondrial (phospho-)proteome might play an important role in resistance to H/R stress of oysters ensuring mitochondrial integrity and function during oxygen fluctuations. This study provides insights into the potential role of proteomic shifts in adaptive response to H/R stress and serves as an important benchmark to understand the mechanisms underlying mitochondrial sensitivity to hypoxia (ischemia) and reoxygenation.

2 Einleitung und Ziele des Projektes

Hypoxia (i.e. oxygen deficiency) is a common stressor in coastal environments caused by the nutrient (N and P) overload, and a major environmental problem in coastal seas. Coastal hypoxia is enhanced by the global climate change that results in warming of the surface waters (thereby lowering O_2 solubility) and expansion of the oxygen minimum zones. Many aquatic organisms also experience short-term hypoxia due to the daily fluctuations of oxygen content in the water as well as the tidal cycles of immersion. Fluctuations in oxygen concentrations are intrinsically stressful to aerobic organisms, due to the decline in the ATP synthesis capacity in hypoxia and elevated production of reactive oxygen species (ROS) during reoxygenation. In highly aerobic terrestrial organisms such as birds and mammals, hypoxia is uncommon and if occurs, is usually damaging or lethal (for example, during ischemia or stroke). Yet many marine invertebrates are adapted to survive prolonged hypoxia and can restore their metabolic capacity (including the mitochondrial function and ATP

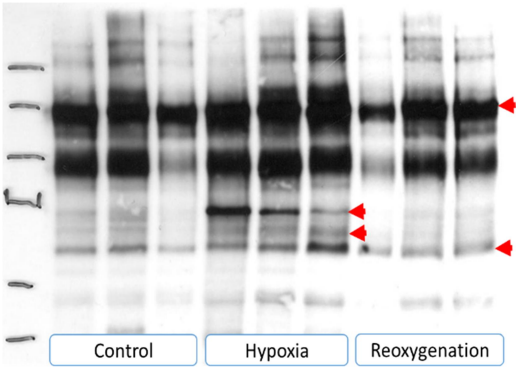

Fig. 1. Effects of H/R stress on phosphorylation profile of mitochondrial proteins of oysters. Mitochondrial proteins phosphorylated on serine and threonine residues were detected by immunoblotting. Red arrows indicate the proteins that are more highly phosphorylated in hypoxia compared to control or reoxygenation.

synthesis) after reoxygenation. To date, the mechanisms responsible for such mitochondrial resilience to hypoxia are not well understood.

Phosphorus (P) is a key element involved in energy transduction (due to its role in formation of ATP), cell signaling, and regulation of metabolic pathways. Specifically, reversible phosphorylation of proteins (i.e. addition of phosphate groups to select amino acids) plays a central role in cellular regulatory networks, switching enzymes and receptors 'on' and 'off' in a highly control manner. Earlier studies showed that reversible phosphorylation regulates glycolysis (i.e. anaerobic breakdown of carbohydrates) during hypoxia (Storey, 2015) but its role in the regulation of mitochondrial function is not well understood. Proteome rearrangements are a common response to environmental stress (Tomanek, 2011) including hypoxia and reoxygenation (Bosworth et al., 2005; Fields et al., 2014; Jiang et al., 2009; Mukherjee et al., 2013). Studies in mammalian models showed that H/R stress affects the abundance of proteins involved in aerobic respiration and antioxidant defense (Eismann et al., 2009; Groehler et al., 2018), induces expression of alternative isoforms of cytochrome c oxidase (COX) (Fukuda et al., 2007; Schiffer et al., 2016) and results in post-translational modifications of proteins involved in cell signaling, glycolysis, ion transport and protein synthesis (Cowan and Storey, 2003; Koumenis et al., 2002; Zhou et al., 2004). However, to date, investigations on proteome plasticity in response to H/R stress have almost exclusively focused on the total cellular proteome (Fields et al., 2014; Gedik et al., 2017; Jiang et al., 2009; Mukherjee et al., 2013; Tomanek, 2011), and organelle-specific proteome rearrangements during H/R stress have not been extensively analyzed. Due to the critical regulatory, signaling and bioenergetics role of mitochondria (Galli and Richards, 2014; Schönenberger and Kovacs, 2015; Sokolova, 2018), understanding shifts in the mitochondrial proteome and phosphoproteome during H/R stress can shed important new light on the mechanisms of metabolic regulation and cellular homeostasis during oxygen fluctuations and identify the potential pathways involved in tolerance to intermittent hypoxia.

In this project, we investigated reversible phosphorylation of mitochondrial proteins as a candidate mechanism for regulating mitochondrial metabolism during hypoxia-reoxygenation (H/R) stress. Our pilot study showed that H/R stress modifies the phosphorylation profile of mitochondrial proteins in a hypoxia-tolerant marine mollusk (Fig. 1) indicating that reversible phosphorylation is involved in metabolic transitions during H/R stress. However, this pilot approach did not permit identification of the specific proteins involved in phosphorylation-dependent metabolic control during H/R stress in mitochondria. Therefore, we used proteomics to identify the mitochondrial proteins that are differentially phosphorylated during H/R stress in organisms with the different degree of hypoxia tolerance. We focused on two species – a hypoxia-tolerant marine mollusk, the Pacific oyster *Crassostrea virginica*, and a hypoxia-sensitive terrestrial vertebrate, the domestic pig. In oysters, we used gill tissues since this is the main organ of gas exchange and the first tissue to encounter ambient oxygen fluctuations in oysters (Kennedy et al., 1996). In pigs, we analysed two different tissues – an extremely hypoxia-sensitive brain and the less hypoxia-sensitive skeletal muscle. At the time of completion of this project, we have obtained the data on the changes in mitochondrial total proteome and phosphoproteome during H/R stress in oysters. We also collected mitochondrial samples from the brain

(thalamus) and muscle (masseter muscle) of pigs immediately after slaughter (controls) and after exposure of isolated tissues to hypoxia. Pig tissue was collected at Leibniz FBN Pigs were slaughtered by electron anesthesia and subsequent exsanguination. The average age of the 4 female animals was 160 days. Mitochondria of control samples were isolated immediately after slaughter. Brain and muscle samples were exposed to hypoxia for 2 hours at 4 °C. Mitochondria were isolated and performed as described elsewhere (Garcia-Cazarin et al., 2011), in brief, 1 g tissue mass was immediately immersed in mitochondrial isolation buffer, homogenized, centrifuged and supernatant disposed; washing of the pellets were repeated three times. The isolation buffer comprises 1% phosphatase inhibitor and 1% protease inhibitor for the isolation of mitochondrial (phosphor-)proteins. Finally, the mitochondrial protein was determined according to Bradford (Bradford, 1976). A total of 16 mitochondrial samples from brain and muscle of 4 German Landrace pigs will be further analyzed. These mitochondria will be analysed in a follow-up project within the Leibniz P-Campus (see section 6).

3 Material und Methoden

Oysters were exposed to hypoxia (<1% O_2) for 24 h at 15°C and salinity 30. For reoxygenation, a subset of oysters exposed to 24 h of hypoxia was placed into a fully aerated aquarium for 1 h. Mitochondria were isolated from gill tissues of oysters exposed to control conditions, hypoxia and reoxygenation. In each experimental group (control, hypoxia and reoxygenation), 6 oysters were used for measurements of mitochondrial respiration, 3 oysters – for total mitochondrial proteome analyses and 5 oysters – for analyses of mitochondrial phosphoproteome. Mitochondrial isolation and functional assays (determination of oxygen consumption and mitochondrial membrane potential) were conducted as described elsewhere (Sokolov and Sokolova, 2018). To extract the total proteome, mitochondrial suspensions were subjected to sonication (2 x 25 sec; Sonoplus HD2070 sonication probe, Bandelin, Berlin, Germany) to disrupt the mitochondrial membranes. Protein concentration in the lysates was determined according to Bradford (Bradford, 1976) using Roti-Nanoquant reagent (Carl Roth, Karlsruhe, Germany). Phosphorylated proteins were isolated from mitochondrial isolates by means of affinity chromatography using Pro-Q® Diamond Phosphoprotein Enrichment Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol for non-denatured proteins. This method enables efficient, nonradioactive isolation of phosphoproteins from complex cellular extracts using phosphoprotein-binding resin that allows efficient capture of both native and denatured phosphoproteins. Total and phosphorylated mitochondrial proteins were analysed at the proteomics facility of the University of Greifswald. The effects of hypoxia and reoxygenation on mitochondrial functional traits were tested using ANOVA and Fisher's least significant differences (LSD) tests for planned contrasts. Normalized abundances of all mitochondrial proteins and enriched phosphoproteins were analyzed using the package randomForest (Liaw and Wiener, 2002) implemented in R (R-Core-Team, 2017) to find proteins that are relevant for differentiation between the three conditions (C, H, R).

4 Ergebnisse

Overall, proteomics analysis of total and phosphoprotein-enriched mitochondrial fractions of oysters identified 422 mitochondrial proteins. Oyster mitochondria show considerable changes in the mitochondrial total and phosphoproteome during H/R stress, encompassing ~50 proteins (>10% of the total proteome) involved in OXPHOS, substrate catabolism, mitochondrial quality control, protein homeostasis, and iron and redox homeostasis, yet very little change in mitochondrial function. This indicates that proteome rearrangement might serve as a basis for the mitochondrial robustness in this hypoxia-tolerant intertidal bivalve, resulting in the adaptive shifts that protect against H/R-induced injury and dysfunction.

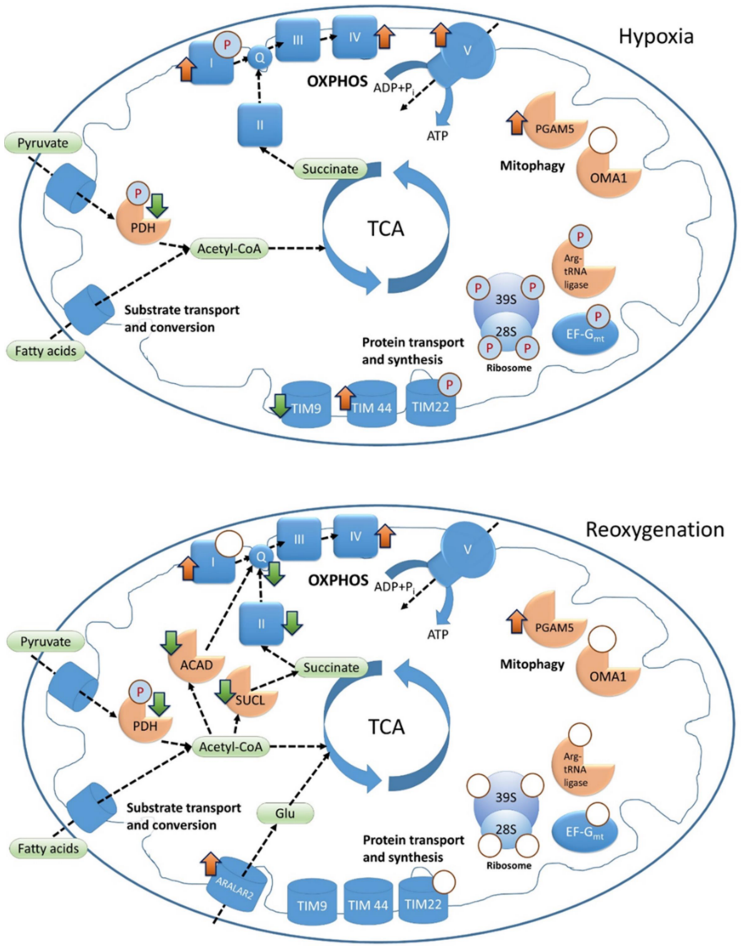

Fig.2. Changes in the abundance and phosphorylation status of proteins involved in mitochondrial OXPHOS, protein transport and synthesis and quality control caused by hypoxia and reoxygenation in gill mitochondria of *C. gigas*.

Filled circles with P indicate proteins whose phosphorylation levels increased, and empty circles indicate proteins whose phosphorylation levels decreased under the respective conditions. Short thick upward and downward arrows indicate proteins that increased or decreased in abundance compared with the control, respectively. Abbreviations: Q – ubiquinone; TCA – tricarboxylic acid cycle; PDH – pyruvate dehydrogenase; 39S and 28 S – 39S and 28S subunits of ribosome; EF-Gmt – mitochondrial elongation factor G; OMA1 - metalloendopeptidase OMA1; PGAM5 - Serine/threonine-protein phosphatase PGAM5; TIM9, TIM44 and TIM22 - mitochondrial import inner membrane translocase subunits 9, 44 and 22, respectively. Thin broken arrows show the flow of electrons and/or substrates.

5 Diskussion

Mitochondria of a hypoxia-tolerant intertidal bivalve *C. gigas* showed functional robustness (indicated by the ability to maintain normal ETS and OXPHOS capacity as well as the mitochondrial membrane potential) during H/R stress, similar to other hypoxia-tolerant bivalves and fish and unlike hypoxia-sensitive invertebrate and vertebrate species, where reoxygenation results in mitochondrial injury and functional collapse [review in (Sokolova, 2018)]. Functional stability of oyster mitochondria during H/R stress goes hand-in-hand with significant rearrangements of the mitochondrial proteome and phosphoproteome that start in hypoxia but become considerably more pronounced during reoxygenation, affecting

a broad range of pathways (Fig. 2). The observed patterns of protein up- and down-regulation during reoxygenation in oyster mitochondria indicate that post-hypoxic recovery is not a passive restoration of the normoxic status quo, but an active regulation responding to the unique physiological challenges caused by reinstated O_2 influx. These rearrangements involve upregulation of mitochondrial ETS proteins (most notably Complexes I and IV), suppression of pathways channeling electrons to ubiquinone, stimulation of mitochondrial quality control mechanisms and modulation of protein synthesis and transport. Overall, our study indicates that shifts in the mitochondrial proteome may play an important role in responses to intermittent hypoxia potentially underlying the functional mitochondrial reorganization elicited by H/R stress (Ivanina et al., 2016; Ivanina and Sokolova, 2016; Kurochkin et al., 2009) and complementing adaptive shifts in anaerobic metabolism and metabolic rate depression (Hochachka, 1993; Ivanina et al., 2010; Sokolova et al., 2011).

The results of this study provided pilot data that led to a successful DFG grant proposal submission (Project MitoBOX, start date 01.02.2019) and laid out the basis for a follow up collaborative proposal between Uni Rostock, Leibniz IOW and Leibniz FBN in the framework of the new 'Molecular Biology of Phosphorus' cluster of the 2nd funding phase of the Leibniz P-Campus.

6 weitere Leistungen aus dem Projekt*

Peer-reviewed publications

1. Sokolov EP, Sokolova IM (2018) Compatible osmolytes modulate mitochondrial function in a marine osmoconformer *Crassostrea gigas* (Thunberg, 1793) . *Mitochondrion* https://doi.org/10.1016/j.mito.2018.02.002
2. Sokolov, E.P., Markert, S., Hinzke, T., Hirschfeld, C., Becher, D., Ponsuksili, S., Sokolova, I.M. (2018) Effects of hypoxia-reoxygenation stress on mitochondrial proteome and bioenergetics of the hypoxia-tolerant marine bivalve Crassostrea gigas. *J Proteomics*. doi: 10.1016/j.jprot.2018.12.009.

Presentations

1. Sokolova IM, Sokolov EP, Ivanina AV (2018) Mitochondria from Hell: The Role of Mitochondrial Mechanisms In Stress Tolerance Of Animal Extremophiles. An invited talk at the symposium. Invited talk at the symposium "Inside the Black Box: The Mitochondrial Basis of Life-History Variation and Animal Performance" of the annual meeting of the Society of Integrative and Comparative Biology, January 2018, San Francisco, CA, USA.
2. Sokolova IM, Sokolov EP (2018). Effects of compatible osmolytes on mitochondrial functions of a marine osmoconformer. Annual meeting of the Society of Integrative and Comparative Biology, January 2018, San Francisco, CA, USA. Poster.
3. Sokolova IM (2018). Mitochondrial responses and tolerance to environmental stress in animal extremophiles. Invited talk at the annual meeting of the symposium 'Mitochondria in changing climates: biosensors and mediators of animal resilience' at the annual meeting of the Society for Experimental Biology, July 2018, Florence, Italy.
4. Sokolov EP, Markert S, Hinzke T, Sokolova IM (2019). Proteomic rearrangements underlie mitochondrial responses to intermittent hypoxia in a hypoxia tolerant

marine bivalve *Crassostrea gigas*. Annual meeting of the Society of Integrative and Comparative Biology, January 2019, Tampa, FL, USA. Poster.

5. Sokolova IM. (2019). Mitochondrial adaptations to fluctuating oxygen levels in hypoxia-tolerant marine bivalves. Invited talk at the symposium "Beyond the Powerhouse: Integrating Mitonuclear Evolution, Physiology, and Theory in Comparative Biology" at the annual meeting of the Society of Integrative and Comparative Biology, January 2019, Tampa, FL, USA.

External funding proposals

1. DFG project "MitoBOX: The mitochondrial basis of hypoxia tolerance in marine mollusks", 01.02.2019-31.01.2022. PI: Sokolova IM (Uni Rostock), co-PI: Bock CB (Helmholtz AWI). Supported.
2. Project 'MetaPhos: Phosphorus as a metabolic regulator during environmental stress in animals' in the framework of the 2nd funding phase of Leibniz P-Campus. PIs: Sokolov EP (Leibniz IOW), Sokolova IM (Uni Rostock), Siriluck Ponsuksili (Leibniz FBN), Wimmers K (Leibniz FBN). Supported.

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Danksagung

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Anhang

Attachment 1. PDF reprint of Publication 1 from the MitoP Project (Sokolov and Sokolova, 2018, Mitochondrion)

Attachment 2. PDF reprint of Publication 2 from the MitoP Project (Sokolov et al, 2018, Journal of Proteomics)