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The morphological and functional significance of the NOS/NO system in the respiratory, osmoregulatory, and contractile organs of the African lungfish

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ABSTRACT

This review aims to summarize the changes of the NOS/NO system which occur in the lungs, gills, kidney, heart, and myotomal muscle of air breathing fish of the genus *Protopterus*, i.e. *P. dolloi* and *P. annectens*, in relation to the switch from freshwater to aestivation, and vice-versa. The modifications of NOS and its partners Akt and Hsp-90, and HIF-1 α , detected by immunohistochemical and molecular biology methods, are discussed together with the apoptosis rate, evaluated by TUNEL. We hypothesize that these molecular components are key elements of the stress-induced signal transduction/integration networks which allow the lungfish to overcome the dramatic environmental challenges experienced at the beginning, during, and at the end of the dry season.

1. Introduction

Due to its ubiquitous occurrence in the animal kingdom and its multilayered signal transduction properties, Nitric Oxide (NO) is involved in a variety of fundamental physiological processes at all levels of biological organization from cell to the whole organism (Moncada and Higgs, 1991). It acts not only as a major autocrine and paracrine modulator, but also as a very diffusible endocrine substance at sites that are distant from its generation thanks to a conversion to the more stable circulating nitrite (Gladwin, 2005; Angelone et al., 2012). NO is involved in the regulation of oxygen consumption, mitochondrial respiration, redox balance, cell survival and osmoregulation (Rassaf et al., 2007; Fago and Jensen, 2015). The relevant cardiovascular functions of NO include the vasomotion of different circuits with the consequent redistribution of blood depending from various physiological and pathophysiological conditions (Moncada and Higgs, 1991). As illustrated in a number of phylogenetically divergent vertebrates, all these actions are very conserved, being crucial in the striking morpho-functional remodeling experienced by species which face often dramatic developmental and/or environmental changes during their lifecycle. A remarkable example is represented by fish species that switch periodically

from water to air-breathing and vice-versa, i.e. a condition in which, among other compensations (including oxygen consumption and redox balance), the organism must orchestrate distinct branchial and pulmonary morpho-functional rearrangements (Zaccone et al., 2016).

The African freshwater lungfish species, *Protopterus dolloi*, *Protopterus aethiopicus*, *Protopterus annectens* and *Protopterus amphibious*, are obligate air breathers having true lungs and reduced gills (Burggrenn and Johansen, 1986; Graham, 1997). During the fry hot summer, they are able to enter in aestivation (i.e. a dormancy state of corporal torpor), thereby surviving for a long period (up to six years in the case of *P. amphibious*) (Lomholt, 1993). To face the prolonged periods of water and food deprivation in a hot environment, the aestivating fish, either in air or in mud, encases itself into a completely dried mucus cocoon (see for review Ip and Chew, 2010; Fishman et al., 1986; Greenwood, 1986). Aestivation, which can be sustained until the moment when water becomes once again available in the habitat, is characterized by the complete dependence on aerial gas exchange, dehydration, metabolic depression, and down-regulation of respiratory and cardiovascular activities (Fishman et al., 1986). The internal stores, i.e. proteins and aminoacids, undergo mobilization while ammonia is detoxified to urea (Storey and Storey, 1990; Perry et al., 2008). In order

Abbreviations: NOS, nitric oxide synthase; NO, nitric oxide; eNOS, endothelial NOS; p-eNOS, phospho-eNOS; nNOS, neuronal NOS; iNOS, inducible NOS; Akt, protein kinase B, phospho-Akt; HIF-1 α , hypoxia inducible factor α ; Hsp-90, heat shock protein 90; FW, freshwater; 6mAe, 6 months of experimentally induced aestivation; 6mAe6d, 6 days after arousal; EE, endocardial-endothelial; GFR, glomerular filtration rate; GBM, glomerular basal membrane; ARC, apoptosis repressor with caspase recruitment domain; Cx43, connexin 43; ROS, radical oxygen species; COX, cyclooxygenase

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to face the drastic environmental challenges, *Protopterus* spp. undergo morpho-functional rearrangements at several levels. This implies a complex interplay between the downregulation and the upregulation of diverse tissue and organ activities. As paradigms of these rearrangements, we will consider here the lungs, the gills, the kidney, the heart, and the myotomal muscle.

Until recently, the role of NO in the compensatory adjustments experienced by the lungfish during its lifecycle has remained completely unknown. However, in the last years, evidence from our and other laboratories, showed in two species of *Protopterus*, i.e. *P. dolloi* and *P. annectens*, that the Nitric Oxide Synthase (NOS) system is involved in the switch from active freshwater life (FW) to aestivation and vice-versa. In this review we will summarize the functional significance of the NO signalling in the lungfish, using the lungs, the gills and the kidney as paradigms of respiratory and osmoregulatory organs, respectively, and the heart and the skeletal muscle as paradigms of contractile tissues.

2. The NOS/NO system in fish: evolutionary and methodological aspects

In fish, as in mammals, NO is produced by NOS by using L-arginine as substrate. Three different NOS isoforms have unambiguously been recognized in mammals: NOSI or neuronal NOS (nNOS), NOSII or inducible NOS (iNOS), NOSIII or endothelial NOS (eNOS) (Andreakis et al., 2011 and references therein). The terms *neuronal* and *endothelial* NOS have only a historical significance due to the first identification of the two isoforms in nerve and endothelial tissues, respectively, but it is now clearly established that they are both expressed in a great variety of cell types. In contrast to the knowledge related to higher vertebrates, there is still little consensus on the types of NOS isoenzymes present in fish. Based on comparative genomic analyses, it has been suggested that fish possess nNOS and iNOS, but not eNOS (Andreakis et al., 2011). In some fish species (e.g. cyprinids), the iNOS sequence has a myristoylation site, which is characteristic of eNOS (Andreakis et al., 2011). At the same time, piscine nNOS isoforms include an endothelial-like consensus, thus covering some functional behaviors typical of eNOS (Andreakis et al., 2011). Interestingly, physiological, and biochemical evaluations, as well as immunological data obtained by our group by using heterologous mammalian anti eNOS antibodies, revealed in several teleost species (i.e. the eurytherm *Anguilla anguilla*, *Tunnus thynnus thynnus* and *Carassius auratus*, and the Antarctic *Chionodraco hamatus* and *Trematomus bernacchii*) the presence of an endocardial-endothelial (EE) NO production that is crucially involved in cardiac modulation (Imbrogno et al., 2010). As extensively described in the present review, an eNOS-like enzyme is also expressed in the lungs, gills, kidneys, heart and skeletal muscles of the lungfish species *P. dolloi* and *P. annectens* (Amelio et al., 2008, 2013a; Garofalo et al., 2015). At the moment a dipnoan NOSIII sequence is unknown. However, it has been recently argued that lungfish possess many tetrapod-like gene sets (for ref see Donald et al., 2015), suggesting that the eNOS isoform could be present in this group. On these premises, we will tentatively refer here as eNOS the eNOS-like enzyme detected in lungfish.

In lungfish we observed that the expression of the putative eNOS isoform is modulated by exposure to the environmental stresses of aestivation and arousal, and is accompanied by changes in the expression of the eNOS partner proteins, Akt (also named Protein Kinase B: PKB) and Hsp-90. As largely shown in mammals, and schematically depicted in Fig. 1, Akt and Hsp-90 act as molecular modulators of eNOS, the first inducing enzyme activation by phosphorylation, the second acting as an adaptor between Akt and eNOS. In a wide variety of homeostatic processes, Akt/Hsp-90/eNOS association represents a key element for the amplification of extracellular signals and their integration with the cellular effector (Amelio et al., 2013a).

3. The experimental African lungfish

The studies summarized in the present paper have taken advantage of the possibility to use laboratory aestivation as a validated standard and repeatable procedure to mimic the natural events that characterize the lifecycle of two species of *Protopterus*, i.e. *P. dolloi* and *P. annectens* as described by Chew et al. (2004). According to these authors, fish were induced to aestivate in an aquarium containing a thin layer of water; in few days, after water dried, the active FW animal entered in torpor within a layer of dried mucus. Under these conditions, lungfish aestivated for different periods, until water was provided again. Specifically, *P. dolloi* was maintained for either a very short (6 days of aestivation: 6DA) or a longer (40 days of aestivation: 40DA) period in laboratory aestivation, while *P. annectens* was induced to aestivate in air for a very long time (6 months of aestivation: 6mAe). After each experimental period a group of fish was sacrificed for sample collection while another was placed in water and sacrificed 6 days after arousal (6mAe6d). These procedures allowed to analyse not only the switch from FW to aestivation, and the maintenance of a brief quiescent phase (*P. dolloi*), but also the recovery after very long periods of inactivity and dormancy (*P. annectens*).

4. Gills vs lungs

During aestivation, gill function is to large extent suppressed, while the lungs become physiologically hypertrophic and extensively vascularized, being the only source available for oxygen supply (Garofalo et al., 2015). Systemic blood is distributed to the pulmonary circulation, where it is oxygenated, and hence to the systemic circuit (Laurent, 1996; Icardo et al., 2016). At the same time, the morphological integrity of the gills (see Ref. 14 in Sturla et al., 2002) is preserved to promptly reestablish their respiratory activity upon arousal. Presently, it is not known how this interplay is orchestrated and what are the signal transduction mechanisms implicated in these rearrangements. Our study (Garofalo et al., 2015.) identified in the branchial and pulmonary tissues of *P. annectens*, eNOS, Akt, Hsp-90, and HIF-1 α . These molecules are important in controlling redox balance, cell survival, and osmoregulatory signal transduction, all processes fundamental for lungfish homeostasis. Moreover, NO is a major paracrine and endocrine vasoactive modulator and, therefore, it can also play a crucial role in the redistribution of blood that must match the distinct branchial and pulmonary remodeling experienced by the FW and the aestivating lungfish, respectively. A recent physio-pharmacological study (Filogonio et al., 2017) showed that NO vasodilates the single *ductus arteriosus* of *P. aethiopicus* (Donald et al., 2015), pointing to a NO-dependent blood flow redistribution between systemic and pulmonary circulation.

We showed in both the gills and lungs of *P. annectens* that eNOS, Akt and Hsp-90 changed in parallel with the aquatic-aerial transition and the arousal in water. In the lungs of fish kept in FW (Fig. 2A) or undergoing aestivation, a strong eNOS signal was evidenced in both capillary endothelial cells and respiratory epithelium, the fluorescence being stronger during aestivation (Fig. 2B). This data is in agreement with eNOS detections in both pulmonary epithelial and endothelial cells of rats (Zhan et al., 2003) and humans (Ricciardolo, 2003). In mammalian epithelial cells, eNOS-produced NO is implicated in ciliary motility (Kim et al., 2001), production and composition of airway surface liquid, and smooth muscle dependent bronchomotility (Ricciardolo, 2003). In endothelial cells it has been suggested that NO exerts a tonic modulation of the pulmonary circulation, counteracting, at the same time, the hypoxic vasoconstriction (Persson et al., 1990).

In *P. annectens* we observed that, in both FW and aestivating fishes, in addition to the endothelium and the epithelium, a weak reticular eNOS labeling is present also in the *septa* that separate individual air spaces of the lungs and are known to contain smooth muscle cells housed in niches (Fig. 2A,B). In the pulmonary *septa* of the lungfish,

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