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Purinergic signaling as a potential target of hypoxia stress-induced impairment of the immune system in freshwater catfish *Lophiosilurus alexandri*

Matheus D. Baldissera[a,*], Carine F. Souza[b], Tulio P. Boaventura[c], Cintia L. Nakayama[c], Bernardo Baldisserotto[b], Ronald K. Luz[c,*]

[a] Department of Microbiology and Parasitology, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Department of Physiology and Pharmacology, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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ABSTRACT

Purinergic signaling plays an important role in the immune and inflammatory responses through the hydrolysis of adenine nucleotides, such as adenosine triphosphate (ATP), and nucleosides, such as adenosine, which are involved in physiological and pathological events as pro-inflammatory and anti-inflammatory mediators. The aim of this study was to evaluate whether purinergic signaling can modulate the immune and inflammatory responses in the plasma of *Lophiosilurus alexandri* exposed to hypoxia. Plasma ectonucleoside triphosphate diphosphohydrolase (NTPDase) activity using ATP as a substrate decreased after 24 and 72 h of hypoxia exposure compared with the normoxia group, while no difference was observed for NTPDase (using ADP as a substrate) and 5′-nucleotidase activities. On the other hand, adenosine deaminase (ADA) activity increased after 24 and 72 h of hypoxia exposure compared with the normoxia group. Moreover, is important to emphasize that enzymatic activity of NTPDase (using ATP as a substrate) and ADA did return to control levels only after a 72 h recovery period. Also, plasmatic levels of pro-inflammatory cytokines (interleukin-1, interleukin-6 and tumor necrosis factor-alpha) and ATP increased after 24 and 72 h of hypoxia exposure compared with the normoxia group. Based on this evidence, our findings reveal that adenine nucleotide hydrolysis is not able to modulate the immune and inflammatory responses of fish exposed to hypoxia stress. Moreover, the downregulation of plasma NTPDase activity develops a pro-inflammatory profile due to the excessive ATP content in the extracellular medium elicited by interaction with the P2X7 purineceptor. In summary, purinergic signaling displays a pro-inflammatory profile in the plasma of *L. alexandri* exposed to hypoxia.

1. Introduction

Dissolved oxygen (DO) is considered one of the most important environmental factors associated with suitable physiological function of various species of freshwater fishes (Zhao et al., 2018), including the freshwater "pacamã" *Lophiosilurus alexandri*. According to Gilmore et al. (2018), low oxygen levels in the aquatic environment, known as hypoxia, occur when DO in the water goes below a level that can sustain the life of an organism and its natural capacity for suitable physiological function, and hypoxia has been considered a critical stressor for fish. Fish exposed to hypoxia may impair growth, reproduction, and immune responses (Díaz and Rosenberg, 2011; Magnoni et al., 2018), culminating in augmentation of fish mortality (Breitburg et al., 2009). Some evidence has suggested a relationship between hypoxic stress and impairment of immune and inflammatory responses (Cheng et al., 2002; Choi et al., 2007), but the pathways involved in this relationship remain poorly understood. Thus, more studies are needed to understand the effects of hypoxia in the fish immune system, such as the involvement of enzymes important in purinergic signaling: ectonucleoside triphosphate diphosphohydrolase (NTPDase), 5′-nucleotidase, and adenosine deaminase (ADA), which play an essential role in the regulation of the immune and inflammatory responses (Burnstock and Boeynaems, 2014; Burnstock, 2016), including during hypoxia in mammals (Pimentel et al., 2013; Losenkova et al., 2018).

Purinergic signaling contributes to the fine tuning of inflammatory and immune responses to efficiently eliminate the danger to the host

* Corresponding authors.
E-mail addresses: mdbaldissera@mail.ufsm.br (M.D. Baldissera), luzrk@vet.ufmg.br (R.K. Luz).

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with minimal damage to healthy tissues (Burnstock, 2016), being able to modulate these responses via interaction of purine nucleotides and purine nucleosides located in the plasma membrane of cells, such as adenosine triphosphate (ATP) and adenosine (Ado) (Chiu and Freund, 2014). Purinergic enzymatic regulation initiates with NTPDase activity, which hydrolyzes ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP), while AMP is then hydrolyzed into Ado by 5′-nucleotidase. Finally, Ado is deaminated by ADA into inosine (Idzko et al., 2014). During physiological conditions, ATP is almost exclusively present inside the cells at millimolar concentrations, while it is practically negligible and at low nanomolar concentrations in the extracellular environment, making ATP a signal of damage, known as a damage-associated molecular pattern (DAMP) (Di Virgilio and Vuerich, 2015). During the inflammatory process, as induced by hypoxia (Biddlestone et al., 2015), ATP is released by necrotic cells into the extracellular environment acting as a potent DAMP, which interacts with specific purineceptors (type P2X7). This interaction induces excessive production and release of inflammatory mediators coupled to an elevated incidence of apoptotic and necrotic cell death due to the release of large amounts of ATP, leading to a self-sustained pro-inflammatory deleterious cycle (Savio et al., 2018). By contrast, Ado provides a protective cellular environment through interaction with type P1 purineceptors, which induce the production of anti-inflammatory mediators (Borowiec et al., 2006). In this sense, Pimentel et al. (2013) demonstrated that modulation of the purinergic system exerts an anti-inflammatory profile in the cerebral cortex of newborn rats exposed to hypoxia-ischemia. Additionally, Losenkova et al. (2018) demonstrated that modulation of the purinergic cascade exerts a protective cellular environment to reduce the amount of ATP in the extracellular environment using culture of endothelial cells exposed to hypoxia.

Based on this evidence, the aim of this study was to evaluate whether purinergic signaling can modulate the immune and inflammatory responses in the plasma of *L. alexandri* exposed to hypoxia.

2. Materials and methods

2.1. Animals and environmental acclimation

Juvenile *L. alexandri* were acclimated for 13 days in the Aquaculture Laboratory (Laqua) of the Veterinary School at the Federal University of Minas Gerais. The fish were maintained in 200 L tanks in a recirculating aquaculture system (RAS) and fed twice a day with extruded commercial diet (32% crude protein, 4–6 mm diameter, Acqua Pesca®). The animals were divided into two RASs, RASA and RASB. Each RAS was composed of mechanical and biological filters installed in a 200 L sump tank, with a 200 W heater thermostat, submersible pump (3000 L per hour). Water quality parameters were evaluated and remained similar in both RAS groups as follows: 27.93 ± 0.67 °C, pH 8.48 ± 0.12, and DO 6.80 ± 0.20 mg/L for RASA, and 27.85 ± 0.61 °C, pH 8.31 ± 0.12, and DO 6.80 ± 0.18 mg/L for RASB.

2.2. Animals and experimental study

Juvenile *L. alexandri* were used to evaluate plasma NTPDase, 5′-nucleotidase, and ADA activities. Each RAS received 36 animals/treatment that were randomly distributed in three tanks (12 animals each, i.e., triplicates). Mean body weight and length of each group was: 50.18 ± 10.37 g and 16.16 ± 1.37 cm for RASA group, and 50.34 ± 12.44 g and 16.42 ± 1.62 cm for RASB group.

In the RASB (hypoxia) group, the aeration and water flow were kept switched off until the DO level reached 2.5 mg/L, and then it was switched on with a minimum flow to maintain the DO level between 2.0 and 3.0 mg/L. The RASA treatment (normoxia group) was maintained in the same conditions used in the acclimation period. In this period, the water quality parameters for RASA were: 27.72 ± 0.55 °C, pH 8.13 ± 0.01, and DO 6.75 ± 0.16 mg/L, and for RASB: 28.0 ± 0.46 °C, pH 8.11 ± 0.02, and DO 2.07 ± 0.63 mg/L. After 72 h, RASB DO levels returned to the same levels as RASA. During the recovery period, the water quality parameters for RASA were: 27.70 ± 0.75 °C, pH 8.04 ± 0.03, and DO 6.15 ± 0.73 mg/L, and for RASB: 27.70 ± 0.75 °C, pH 8.05 ± 0.04, and DO 6.17 ± 0.68 mg/L. During the experimental study, the animals were fed twice a day until apparent satiety with the commercial diet previously described. Any uneaten food, feces, and other residues were removed daily 30 min after feeding to maintain water quality.

The methodology used in this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Minas Gerais under protocol number 154/2018.

2.3. Sample collection

Blood samples of three juveniles from each tank (n = 9 per treatment) were collected from the caudal vein using syringes containing anticoagulant (sodium heparin) at 24 and 72 h of hypoxia exposure, and at 24 and 72 h of the recovery period. The samples were centrifuged at 1000 × *g* for 15 min at 4 °C to obtain plasma for measuring the NTPDase, 5′-nucleotidase, and ADA activities, as well as the levels of pro-inflammatory cytokines and ATP. The plasma samples were stored at −80 °C until further use.

2.4. Plasma NTPDase and 5′-nucleotidase activities

Plasma NTPDase and 5′-nucleotidase activities were measured as previously described by Oses et al. (2004). A reaction mixture containing 3 mM of ATP or ADP as substrate and 112.5 mM Tris-HCl (pH 8.0) was used to measure NTPDase activity, while 3 mM of AMP as substrate and 100 mM Tris-HCl (pH 7.5) was used to measure 5′-nucleotidase activity. Reaction mixtures were incubated with approximately 2.0 mg of plasma protein at 37 °C for 40 min in a final volume of 200 μL. The reaction was stopped by the addition of 200 μL of 10% of trichloroacetic acid. All samples were centrifuged at 5000 × *g* for 5 min at 4 °C to remove precipitated protein, and the supernatant was used for the colorimetric assay. Samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured according to Chan et al. (1986). Enzymatic activities were expressed as nanomoles of Pi released per min per milligram of protein (nmol of Pi/min/mg of protein).

2.5. Plasma ADA activity

Plasma ADA activity was measured according to the method of Giusti and Gakis (1971), which is based on the direct measurement of ammonia produced by the enzyme in the presence of Ado, using 50 μL plasma samples. The enzymatic reaction was started by the addition of 500 μL of Ado (21 mM) as a substrate and stopped by adding 1500 μL of 106/0.16 mM phenol/sodium nitroprusside to the reaction mixture, which was immediately mixed with 1500 μL of 125/11 mM alkaline hypochlorite solution. Ammonium sulfate (75 μM) was used as the ammonium standard. ADA activity was expressed as U Ado/mg of protein.

2.6. Plasma pro-inflammatory cytokines levels

Interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α) were quantified by ELISA assay using specific commercial Quantikine Immunoassay kits (Genxbio Health Sciences, Delhi, India), according to the manufacturer's recommendations and explained in details by Baldissera et al. (2017). All results were expressed as pg/mL.