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Aeromonas caviae alters the activities of ecto-enzymes that hydrolyze adenine nucleotides in fish thrombocytes

Matheus D. Baldissera[a,*], Carine F. Souza[b], Camila M. Verdi[a], Bruno S. Vizzotto[c], Roberto C.V. Santos[a], Bernardo Baldisserotto[b,**]

[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] Laboratory of Molecular Biology, Centro Universitário Franciscano, Santa Maria, RS, Brazil

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ABSTRACT

It is recognized that the purinergic system, through the activities of ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-5′-nucleotidase (E-5′-nucleotidase), and ecto-adenosine deaminase (E-ADA), is involved in the regulation and modulation of the physiological and pathological events linked to hemostasis. This occurs due to the role of adenosine diphosphate (ADP) in the activation and recruitment of platelets, and the role of adenosine (Ado) in the inhibition of platelet activation. Thus, here we aimed to evaluate whether *Aeromonas caviae* infection impairs the ecto-enzymes of the purinergic system in fish thrombocytes and the involvement of this system in the hemorrhagic septicemia. The total number of fish thrombocytes decreased in infected animals compared to uninfected animals. Regarding the ecto-enzymes of the purinergic system, the E-NTPDase and E-5′-nucleotidase activities increased in infected animals compared to uninfected animals, while the E-ADA activity decreased. These findings show that adenine nucleotide hydrolysis is modified in the thrombocytes of fish experimentally infected with *A. caviae,* which impairs the coagulation process due the excessive hydrolysis of ADP, a molecule linked with activation and recruitment of thrombocytes at the site of vascular injury, and augmentation on Ado levels, a molecule linked with inhibitory effects on platelet activation and aggregation. In summary, the purinergic system might contribute to the occurrence of hemorrhagic frames in fish infected with *A. caviae.*

1. Introduction

Aeromonas caviae is a mesophilic anaerobic Gram-negative bacterium belonging to the Aeromonadaceae family and found in aquatic environments, such as aquacultures, and can infect a vast number of fishes [1], including the silver catfish (*Rhamdia quelen*) [2]. This bacterium is linked to hepatosplenomegaly, eye disease, and ulcerative lesions on the body surface, and has gained clinical recognition as one of the most prevalent causative agents of hemorrhagic septicemia [1]. However, the pathways involved in the occurrence of hemorrhagic septicemia during *A. caviae* infection remain unknown. Therefore, we decided to evaluate the activities of enzymes belonging to the purinergic system in thrombocytes, which play an essential role in the coagulation process [3,4].

The principal role of blood thrombocytes is to ensure primary hemostasis in order to maintain the blood vessel integrity and the rapid cessation of bleeding in the event of loss of vascular integrity [5]. The purinergic system plays an important regulatory role in the coagulation process by extracellular biomolecules, mainly adenosine diphosphate (ADP) and its derivate nucleoside adenosine (Ado) [6]. Nucleotide enzymatic regulation is initiated by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), which hydrolyzes ADP into adenosine monophosphate (AMP), whereas ecto-5′-nucleotidase (E-5′-nucleotidase) hydrolyzes AMP to Ado. Moreover, Ado is cleaved to inosine by the enzyme ecto-adenosine deaminase (E-ADA) [7]. The nucleotide ADP is a physiological platelet activator and recruiter, being stored at high concentrations in platelet-dense granules and released from stimulated platelets to promote platelet activation. These effects are mediated by purinergic receptors, such as P2X1, P2Y1, and P2Y12 [8]. On the other hand, the nucleoside Ado is an important inhibitor of platelet activation and inhibitor of ADP-induced platelet aggregation [9].

Based on this evidence, here we aimed to evaluate whether *A. caviae* infection impairs the ecto-enzymes of the purinergic system in fish

* Corresponding author.
** Corresponding author.
E-mail addresses: mdbaldissera@mail.ufsm.br (M.D. Baldissera), bbaldisserotto@hotmail.com (B. Baldisserotto).

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thrombocytes and the involvement on this system in the hemorrhagic septicemia.

2. Material and methods

2.1. Fish harvesting, maintenance of animals, and water quality parameters

Healthy fish were collected for experimental purposes from a fish farm located in southern Brazil. The animals were analyzed for the absence of ectoparasites or endoparasites in the gills, skin and fins, as well as observed for the absence of skins lesions compatible to possible bacterial infections, that prove the animal's health. The animals were transported to the Laboratório de Fisiologia de Peixes at the Universidade Federal de Santa Maria, where they were maintained in 250-L fiberglass tanks with continuous aeration under controlled water parameters, as recently published in detail by Baldissera et al. [2]. The animals were fed to apparent satiation with commercial feed once per day. Any uneaten food, feces, and other residues were removed daily 1 h after feeding.

2.2. Inoculum confirmation and preparation

The pathogen was confirmed through colony morphology and physiological characteristics, as well as by using polymerase chain reaction (PCR) analysis of the 16S rRNA gene of *A. caviae* using the primers 5′ TCG TTG GGT TGG GAT GTG 3′ (forward) and 5′ TGT TAC CGC GGT GAA AGG 3′ (reverse), according to the methodology described by Baldissera et al. [2].

The bacterial isolate was grown on nutrient agar for use in this experimental model. The suspension of *A. caviae* was washed twice in sterile saline (NaCl 0.9%), turbidity (OD600) was adjusted to 0.9–1.1 (equivalent to 10^6 CFU/mL), and used for the infection model.

2.3. Fish and study design

Twenty adult silver catfish (118 ± 21 g; 30 ± 3 cm) were used as the experimental model to assess the E-NTPDase, E-5′-nucleotidase, and E-ADA activities in thrombocytes. The fish were allocated into two groups of 10 animals each: uninfected animals (negative control group) and experimentally infected animals (positive control group) inoculated intramuscularly with 100 µL of a bacterial suspension containing 55×10^6 viable cells of *A. caviae,* according to the protocol established by Baldissera et al. [2]. The negative control group received the same dose of sterile saline by the same route.

The methodology used in the experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria under protocol number 074/2014.

2.4. Sampling

On day 4 post-infection (PI), blood samples were collected from the caudal vein in tubes containing citrate as an anticoagulant for thrombocytes separation. Thereafter, all animals were anesthetized during approximately 180 s with 300 µL/L of natural anesthetic (*Cymbopogon flexuosus* essential oil) followed by the severing of the spinal cord according to the Ethics Committee recommendations.

2.5. Thrombocyte count and preparation

Blood smears stained by the Romanowsky method were used for thrombocytes count according to the technique described by Withyachumnarnkul et al. [10]. The thrombocytes were prepared as previously described by Pilla et al. [11] and modified by Lunkes et al. [12] for platelets. The blood samples were centrifuged at 160 × *g* for 15 min, and the thrombocyte-rich plasma was centrifuged at 1400 × *g* for 30 min, and washed twice with 3.5 mM HEPES buffer containing 142 mM NaCl, 2.5 mM KCl and 5.5. mM glucose. The washed thrombocytes were resuspended in HEPES to determine the enzymatic activities.

2.6. E-NTPDase and E-5′-nucleotidase activities

The E-NTPDase enzymatic assay was evaluated according to the methodology previously described by Lunkes et al. [13], reported in detail by Souza et al. [3]. Enzyme-specific activities were reported as nmol Pi released/min/mg of protein.

2.7. E-ADA activity

E-ADA activity was determined as previously described by Giusti and Galanti [14], reported in detail by Souza et al. [3]. Enzyme-specific activity was reported as U/mg of protein.

2.8. Statistical analyses

Normality and homoscedasticity were analyzed with the Kolmogorov-Smirnov and Levene tests, respectively. Significant differences between groups were analyzed and detected with a two-tailed Student's *t*-test for independent samples. Differences were considered statistically significant at $p < .05$. The effect size (r^2) was described and scored as follows: ≤ 0.1 (small), 0.1 ≤ 0.3 (medium), and ≥0.5 (large).

3. Results

3.1. Thrombocytes count

The number of thrombocytes decreased by 30% [$t(18) = 2.18$; $p = .033$; $r^2 = 0.35$] in animals infected by *A. caviae* compared with the uninfected control group (Fig. 1).

3.2. E-NTPDase, E-5′-nucleotidase and E-ADA activities

E-NTPDase activity (ADP as substrate) increased by 80% [$t(18) = 4.31$; $p = .0012$; $r^2 = 0.67$] and E-5′-nucleotidase activity increased by 50% [$t(18) = 3.36$; $p = .0025$; $r^2 = 0.42$] in the thrombocytes of animals infected by *A. caviae* compared with the uninfected control group (Fig. 2A and B). On the other hand, the E-ADA activity decreased by 40% [$t(18) = 3.16$; $p = .0032$; $r^2 = 0.39$] in the thrombocytes of animals infected by *A. caviae* compared with the uninfected control group (Fig. 2C).

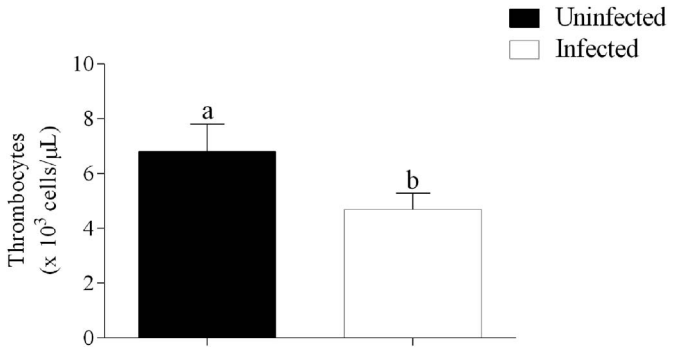

Fig. 1. Absolute number of peripheral thrombocytes in silver catfish experimentally infected with *Aeromonas caviae* compared with the uninfected control group on day 4 post-infection (PI). Bars with different letters are significantly different ($p < .05$; n = 10 per group) using the two-tailed Student's *t*-test for independent samples.