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Neuroendocrine modulation of the inflammatory response in common carp: Adrenaline regulates leukocyte profile and activity

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ABSTRACT

Inflammatory responses have to be carefully controlled, as high concentrations and/or prolonged action of inflammation-related molecules (e.g. reactive oxygen species, nitric oxide and pro-inflammatory cytokines) can be detrimental to host tissue and organs. One of the potential regulators of the inflammatory process are stress mediators including adrenaline. *In vivo* effects of adrenaline were studied during zymosan-induced (Z) peritoneal inflammation in the common carp *Cyprinus carpio* L. Adrenaline injected together with zymosan (ZA) did not change the number of inflammatory leukocytes in the peritoneal cavity, however at 24 h post-injection it significantly reduced the percentage of monocytes/macrophages. Moreover, compared to cells retrieved from fish treated with PBS or zymosan only, adrenaline increased the percentage of apoptotic leukocytes in the focus of inflammation. Furthermore, adrenaline significantly reduced the expression of chemokine CXCL8_L1 (a functional homolog of mammalian IL-8) and its receptors (CXCR1 and CXCR2), indicating changes in leukocyte recruitment after stress.

We conclude that adrenaline may contribute to a coordinated reaction by influencing the inflammatory response via direct regulation of leukocyte migration and/or apoptosis.

1. Introduction

The neuroendocrine and immune systems are linked through bidirectional communication pathways that enable the coordination of physiological, behavioral and immunological responses to changing internal and external conditions [1]. Although the first data indicating effects of stress hormones on the mammalian immune response were described over a century ago, insight into the mechanisms of interaction between the neuroendocrine and the immune systems, especially in ectothermic vertebrates, is still limited.

Teleost fish represent more than one-half of all extant vertebrate species and their ability to continuously adapt to altering circumstances must have contributed to this success. They moreover are intriguing models to study neuroendocrine- immune cooperation, as the head kidney, the fish analogue of the mammalian bone marrow and responsible for haematopoiesis, also harbours the interrenal tissue. This neuroendocrine center is responsible for production and release of adrenaline and cortisol after stressful events [47] and its co-localization with the haematopoitic tissue enables paracrine interactions. Analogue to the situation in higher vertebrates, the fish stress response is characterized by activation of the brain–sympathetic–chromaffin cell axis (BSC) and/or the hypothalamic–pituitary–interrenal axis (HPI). During BSC activation the catecholamines (CA) adrenaline and noradrenaline are rapidly released into circulation from chromaffin cells in the head kidney. Activation of the HPI-axis starts in neurons of the hypothalamic nucleus preopticus (NPO), which release corticotropin-releasing hormone (CRH). CRH stimulates corticotrope cells in the pituitary pars distalis (PD) to secrete adrenocorticotropic hormone (ACTH), which is cleaved from the pro-hormone pro-opiomelanocortin (POMC). ACTH stimulates the release of cortisol from interrenal cells into the blood stream. Pituitary hormones other than ACTH may also play a role in control of cortisol release. Candidates include the POMC derived peptides α-melanocyte-stimulating hormone (α-MSH) and endorphins (END) [16].

Interestingly, both in mammals and in teleost fish, immune organs are innervated by sympathetic neurons. In fish, sympathetic innervation of lymphoid tissue was found in the spleen of coho salmon, where nerve fibers are associated with the vasculature and the melanomacrophage centers [17]. Moreover, immune cells express receptors for stress hormones and neurotransmitters, including adrenergic receptors (ARs). Mammalian innate immune cells express both α- and β-AR subtypes, while on T and B lymphocytes an exclusive expression of adrenergic receptors of the β2 subtype was found [27]. So far, several subtypes of adrenergic receptors have been sequenced in zebrafish [50], trout [29,30] and black

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bullhead catfish [9]. Recently we sequenced the β2a-adrenergic receptor in common carp and showed that it is constitutively expressed not only in neuroendocrine structures and liver, but also in immune organs and leukocytes [7]. Experiments with specific radioligands confirmed expression of functional adrenergic receptors on goldfish [23] and channel catfish leukocytes [14]. Furthermore, high expression of the β2a-AR gene was reported for zebrafish and rainbow trout lymphoid organs (spleen, kidney) [29,50].

Interestingly, both in mammals and in fish, catecholamines may affect immune responses. For example, in mammals *in vitro* administration of adrenaline inhibited the respiratory burst [2,51], reduced the level of pro-inflammatory cytokines (TNF-α and IL-12) e.g. [10,20,39] but promoted the secretion of anti-inflammatory IL-10 [37,43,44]. In fish, adrenergic agonists decreased phagocytosis of rainbow trout and spotted murrel macrophages [28,33]. Furthermore, adrenaline and isoproterenol (βAR agonist) reduced the production of reactive oxygen species (ROS) in rainbow trout pronephric phagocytes [18], while phenylephrine and noradrenaline promoted the respiratory burst in trout and *Channa punctatus* leukocytes respectively. Both ligands generated this effect via α-adrenergic receptors [18,33].

Our recent studies showed that in carp *in vitro* administration of adrenaline reduced the synthesis of ROS and nitric oxide, while it enhanced arginase activity in fish phagocytes. Furthermore, *in vitro* adrenaline inhibited the expression of pro-inflammatory cytokines, chemokines and their receptors. It was therefore hypothesized that adrenaline will down-regulate phagocyte skewing towards classical/innate polarization [7].

One of the best examples of cooperation between the neuroendocrine and immune system is the inflammatory response. The main purpose of inflammation is to transport fluid, proteins, and cells from the blood into the infected and/or damaged tissue. Therefore, the most critical phases of this response are: (i) the increase of local blood vessel permeability, which leads to leakage of plasma into the site of inflammation, (ii) leukocyte migration, followed by elimination of pathogens/cause, and (iii) termination of the reaction and tissue remodeling [26]. Multiple inflammatory mechanisms are already described for fish. In carp, we showed that intraperitoneal injection of zymosan (yeast cell wall components with high concentrations of glucans) significantly induces leukocyte infiltration in the peritoneum and expression of pro-inflammatory mediators like cytokines and chemokines [5]. Interestingly, during zymosan-induced inflammation peritoneal leukocytes show increased expression of genes encoding receptors for stress mediators including receptors for cortisol (GR1a, GR1b), opioids (MOR) and catecholamines (β2a-AR) [4,7,35]. Furthermore, stimulation with morphine significantly reduced the number of inflammatory leukocytes due to reduced expression of chemokines and chemokine receptors [6].

We here investigated the *in vivo* effects of adrenaline on the inflammatory response in common carp.

2. Materials and methods

2.1. Animals

Six month old carp (50–60 g, *Cyprinus carpio* L.) from the Institute of Ichthyobiology and Aquaculture, Polish Academy of Science, Golysz, Poland, were kept at 20 °C in recirculating tap water at the Institute of Zoology facility in Krakow. Fish were fed dry food pellets (Trouvit, Nutreco) at a daily maintenance rate of 1% of their estimated body weight. This experiment was conducted according to license No. 74/2008 from the local ethical committee.

In order to avoid additional stress and/or differences in handling all injections and samplings were performed by the same person and at the same time of day and animals were anaesthetized with 0.2 g l^{-1} tricaine methane sulphonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA), buffered with 0.4 g l^{-1} $NaHCO_3$ (Merck, Darmstadt, Germany). Animals was kept in equally positioned tanks and all unnecessary interference was avoided.

2.2. Zymosan-induced peritonitis

Fish were kept untreated (intact fish, time 0), or they were i.p. injected either with sterile PBS (270 mOsM), freshly prepared zymosan A (Z, 2 mg/ml, 1 ml/50 g b.w., Sigma–Aldrich, St. Louis, MO) in sterile PBS or zymosan together with adrenaline (ZA, 5 mg/kg b.w., Sigma–Aldrich, St. Louis, MO). At 6, 24, 48 or 96 h post injection, animals were sacrificed and peritoneal cavities were lavaged with 2 ml of ice-cold PBS. Total numbers of peritoneal leukocytes (PTL) were determined in a hemocytometer. To determine the cell composition of peritoneal leukocytes, peritoneal fluid samples (24 h after injection) were analyzed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 5000 threshold events per sample were collected and analyzed for their forward scatter (FSC) (for cell size) and sideward scatter (SSC) (cell complexity) profiles. Data were analyzed using WinMDI 2.9 software (Joe Trotter, http://facs.scripps.edu).

2.3. Activity of peritoneal leukocytes

2.3.1. Apoptosis

Apoptotic leukocytes were identified quantitatively by Annexin V-PE Apotosis Detection Kit I (BD Pharmingen) that enables cell staining with Annexin V (binds to phosphatidylserine exposed on the outer membrane of apoptotic cells) and 7-amino-actinomycin (7-AAD) (which enters dead cells). Briefly, cells were washed twice with cold PBS and resuspended in binding buffer. Then 5 μl of Annexin V-PE and 5 μl of 7-AAD were added to the cells (10^5 cells/100 μl binding buffer) and incubated for 15 min at room temperature in the dark. Measurement was performed with a FACSCalibur flow cytometer (with a 15 mW argon ion laser at 488 nm) equipped with CellQuest software (Becton Dickinson, San Diego,

Table 1
Primer sequences with corresponding accession numbers used for gene expression studies.

Gene	Sense (5′–3′)	Antisense (5′–3′)	Acc. No.
40S	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
IL-1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	AJ245635
CXCa	CTGGGATTCCTGACCATTGGT	GTTGGCTCTCTGTTTCAATGCA	AJ421443
CXCb	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB082985
CXCR1	GCAAATTGGTTAGCCTGGTGA	AGGCGACTCCACTGCACAA	AB010468
CXCR2	TATGTGCAAACTGATTTCAGGCTTAC	GCACACACTATACCAACCAGATGG	AB010713
iNOS	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCAGAGTCTCTTCT	AJ242906

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