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Stress differentially affects the systemic and leukocyte estrogen network in common carp

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ABSTRACT

Both systemic and locally released steroid hormones, such as cortisol and estrogens, show immunomodulatory actions. This research gives evidence that circulating and leukocyte-derived estrogens can be involved in the regulation of the immune response in common carp, during homeostasis and upon restraining stress. It was found that stress reduced level of blood 17β-estradiol (E2) and down-regulated the gene expression of components of the "classical" estrogen system: the nuclear estrogen receptors and the aromatase CYP19, in the hypothalamus, the pituitary and in the ovaries. In contrast, higher gene expression of the nuclear estrogen receptors and *cyp19a* was found in the head kidney of stressed animals. Moreover, stress induced changes in the E2 level and in the estrogen sensitivity at local/leukocyte level. For the first time in fish, we showed the presence of physiologically relevant amounts of E2 and the substrates for its conversion (estrone – E1 and testosterone – T) in head kidney monocytes/macrophages and found that its production is modulated upon stress. Moreover, stress reduced the sensitivity of leukocytes towards estrogens, by down-regulation the expression of the *erb* and *cyp19* genes in carp phagocytes. In contrast, *era* expression was up-regulated in the head kidney monocytes/macrophages and in PBLs derived from stressed animals. We hypothesize that, the increased expression of ERα, that was observed during stress, can be important for the regulation of leukocyte differentiation, maturation and migration.

In conclusion, these results indicate that, in fish, the estrogen network can be actively involved in the regulation of the systemic and local stress response and the immune response.

1. Introduction

Neuroendocrine factors can modulate the immune response in lower vertebrates, such as fish, in a manner to that observed in mammals. This serves to provide an effective response to pathogens and to prevent damage of the host tissue [77]. The immunomodulatory action of hormones is rather complex and depends on the physiological status of the organism, e.g. infected vs. non-infected or stress vs. non-stressed. These conditions will alter the cellular composition, the cell type specific microenvironment and the hormone concentrations. The level of circulating hormones is predominantly regulated within specific axes. The estrogen synthesis is regulated by the hypothalamic–pituitary–gonadal axis (HPG). Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus and stimulates the anterior pituitary gland to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The latter hormones stimulate the gonads to produce steroids, including estrogens and androgens. However, the

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synthesis of these reproductive hormones is also influenced by other hormones. For example, the master regulator of the stress axis - corticotropin-releasing hormone (CRH), inhibits the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus [10]. Moreover, recent studies suggest that stress can modulate locally circulating hormone levels, thereby changing the expression of enzymes involved in estrogen production [13]. Local estrogen biosynthesis is critically dependent on testosterone (T) availability. The aromatase cytochrome P450 (CYP19) enzyme regulates its conversion to 17β-estradiol (E2). Moreover, the 17-β-dehydrogenase 1 (HSD17β1) enzyme can convert estrone (E1) to E2, but in the inactivating pathway, HSD17β oxidizes E2 to E1 [33]. CYP19 is also involved in the transformation of androstenedione to E1. Both E1 and T arise from androstendione. This reaction is regulated by aromatase CYP19 and dehydrogenase HSD17β3, respectively. Androstendione originates from the conversion of dehydroepiandrosterone - DHEA (Fig. 1S) [76].

Aromatase is expressed in many extragonadal tissues, including bone, adipose tissue, the prostate and the brain [70]. Previous studies demonstrated that norepinephrine selectively inhibits the aromatase activity in cultured fetal brain of a rat [26], and acute restraint stress can rapidly alter the activity of the aromatase in a direction opposite to that generally induced by sexual interactions [12].

In fish, two aromatase genes were discovered: *cyp19a,* predominantly expressed in ovaries, and *cyp19b*, displaying the highest expression in the brain [17,46,51]. Moreover, *cyp19a* and *cyp19b* show a different response to estrogens since *cyp19b* is up-regulated upon E2-treatment only. These data are consistent with the fact that structural analysis of the *cyp19b* and *cyp19a* gene promoters revealed estrogen response elements (EREs) in the *cyp19b* promoter only [4,75].

Previously, we [72,73,82] and others [78,80,84] reported that steroid hormones such as cortisol and E2, modify the immune response of fish, both *in vitro* and *in vivo*. In mammals, low concentrations of E2, like those occurring during the post-menopausal period, favor T helper 1 (Th1) cell responses and promote cell-mediated immunity and the production of pro-inflammatory cytokines. In contrast, high concentrations of E2 promote a Th2 response, humoral immunity and production of IL-4, IL-5, IL-9 IL-10 and IL-13 [57]. Also for fish, there is evidence that estrogens can affect both the innate immune response (phagocytosis, ROS, NO production and lysozyme activity), and the adaptive response (lymphocyte proliferation and/or activity) (e.g. Refs. [2,3,37,40,64,73]. The strength and direction of the immunomodulatory action of E2 largely depends on the level of expression of the appropriate receptors in/on leukocytes. Both mammalian and fish leukocytes express nuclear steroid receptors such as glucocorticoid (GR), mineralocorticoid (MR), androgen (AR) and estrogen (ER) receptors (e.g. Refs. [25,44,66,71,73]). Recently, we found that in carp leukocytes, both nuclear estrogen receptors (ERα and ERβ), and a surface G-protein coupled estrogen receptor (GPER-1/GPR30) are expressed [73]. Furthermore, both mammalian and fish leukocytes may also be a site of hormone production. For instance, synthesis by fish leukocytes of proopiomelanocortin, giving rise to adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (α-MSH) [49,50], and of corticotropic-releasing factor (CRF) [45] has been revealed. Interestingly, the locally synthesized hormones and the hormones produced in the endocrine glands may function differentially. For example, extragonadal synthesized E2 acts predominantly at local tissue level, in a paracrine or intracrine fashion [16,43,55]. Thus, although the total amount of E2 synthesized by these sites may be small, the local tissue concentrations reach sufficient levels to exert relevant local biological effects [68]. This hormone production is cell-type specific. Schmidt and co-workers [61] showed that human monocyte-derived macrophages, but not monocytes, express aromatase mRNA. Yet, little is known about the role of this local steroid production in the regulation of the immune response.

Also in fish, we [74] and others [24] showed that the aromatase encoding *cyp19* genes are expressed in lymphoid organs and in leukocytes. However, aromatase activity has not yet been studied in different immune cells and tissues. To better clarify a potential role of circulating and leukocyte-derived estrogens in the regulation of the immune response in fish, during homeostasis and upon stress, we now compared the expression patterns of the estrogen receptors and the aromatases in the stress axis organs (HPI, hypothalamus-pituitary-interenal cells of the head kidney), in the HPG organs, as well as in carp leukocytes. We moreover studied the effect of stress on the aromatase activity and determined the levels of locally produced estrogens in monocytes/macrophages.

2. Materials and methods

2.1. Animals

Immature individuals of common carp (*Cyprinus carpio* L., 100 g body weight) were obtained from the Department of Ichthyobiology and Aquaculture, Polish Academy of Science, Golysz, Poland. Prior to the experiments, fish were adapted for four weeks at 20 °C in recirculating tap water at the facility of the Institute of Zoology and Biomedical Research in Krakow, Poland. Fish were kept in equally positioned identical tanks and all unnecessary interferences were avoided. Fish were fed pelleted dry food (Aller Aqua Polska Sp. z o. o., Poland) at a daily maintenance rate of 1% of their estimated body weight.

To avoid additional stress and/or differences in handling, all samplings were performed by the same person and at the same time of day (at 9.00 am). All animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies, and procedures were approved by the Local Ethical Committee (license number 23/2012 and 84/2015).

2.2. Stress model

Prolonged restraint (24 h) was given by netting the fish and suspending the nets with the fish in the tanks as described previously [23,52]. Briefly, fish were stressed by confinement of one fish in a net in their own aquarium while maintaining full contact with water. During the stress challenge fish were not fed. After 24 h, the experimental group was transferred all at once to a tank with 0.2 g l^{-1} Tricaine methane sulphonate (TMS, Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.4 g l-1 NaHCO3 (POCH, Gliwice, Poland), resulting in rapid (<1 min) and deep anesthesia prior to blood sampling and killing. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anesthesia, immediately before sampling of the stress treatment group. Every experiment was performed independently 3 times with at least 3 fish per group every time.

2.3. Blood sampling

Fish were bled through puncture of the caudal vein using a needle attached to a 5-ml syringe. The samples were taken midline just posterior of the anal fin. Every time approximately 5 ml of blood was removed from the caudal vein into a syringe. Blood was collected in covered test tubes and allowed to clot overnight at 4 °C. Blood clots were removed by centrifuging at 3000 × g for 30 min