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Research paper

Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture

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ABSTRACT

A tight interaction between endocrine and immune systems takes place mainly due to the key role of head kidney in both hormone and cytokine secretion, particularly under stress situations in which the physiological response promotes the synthesis and release of stress hormones which may lead into immunomodulation as side effect. Although such interaction has been previously investigated, this study evaluated for the first time the effect of stress-associated hormones together with their receptor antagonists on the expression of cytokine genes in head kidney primary cell culture (HKPCC) of the freshwater rainbow trout (*Oncorhynchus mykiss*) and the seawater gilthead sea bream (*Sparus aurata*). The results showed a striking difference when comparing the response obtained in trout and seabream. Cortisol and adrenocorticotropic hormone (ACTH) decreased the expression of immune-related genes in sea bream but not in rainbow trout and this cortisol effect was reverted by the antagonist mifepristone but not spironolactone. On the other hand, while adrenaline reduced the expression of pro-inflammatory cytokines (IL-1β, IL-6) in rainbow trout, the opposite effect was observed in sea bream showing an increased expression (IL-1β, IL-6). Interestingly, this effect was reverted by antagonist propranolol but not phentolamine. Overall, our results confirm the regional interaction between endocrine and cytokine messengers and a clear difference in the sensitivity to the hormonal stimuli between the two species.

1. Introduction

Stressors may compromise the overall health status in fish including increased susceptibility to pathogens and reduced disease resistance. The stress response, as a general, non-specific and widespread reaction, involves all physiological systems, and particularly the neuroendocrine and immune systems which are tightly connected (Engelsma et al., 2002). In fish, the head kidney plays a principal role in this network since, not only is crucial for the organization of the systemic stress response in fish, secreting corticosteroids and catecholamines, but also for its main role in the immune response as lymphopoietic tissue, and in energetics as the producer and supplier of oxygen carrying red blood cells.

It has been demonstrated that the Hypothalamus-Pituitary-Interrenal (HPI) and Sympathetic Adreno-Medullar (SAM) axes, as the two major pathways by which the endocrine response is organized, modify the immune function in mammals and fish (MacKenzie et al., 2006; Padgett and Glaser, 2003). Cortisol is secreted by head kidney interrenal cells in a concatenated response involving the hypothalamic corticotrophin-releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH) secretion. Thus, secreted ACTH is recognized by melanocrotin receptor 2 (MC2R) on the surface of the interrenal cells and activates a signalling cascade which mediates the secretion of cortisol. Cortisol is the major glucocorticoid (GC) in teleost fish and the final product of HPI axis activation. It plays essential roles in energy homeostasis, including balance maintenance, modulation of the immune response, and regulates behaviour through genomic (slow) and non-genomic (fast) mechanisms in the central nervous system (Castro et al., 2011; Cortés et al., 2013; Mommsen et al., 1999).

Abbreviations: HPI, Hypothalamus-Pituitary-Interrenal; SAM, Sympathetic-Adreno-Medullar; CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone; MC2R, melanocrotin receptor 2; GCs, glucocorticoids; GREs, glucocorticoid response elements; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; AR, adrenergic receptor; HKPCC, head kidney primary cell culture.

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Cortisol activates glucocorticoid receptors (GRs) in responsive cells leading to modulation of target genes expression. Corticosteroids bind to GRs forming a complex that is transported to the nucleus where it binds to DNA at glucocorticoid response elements (GREs) present in the promoters of several genes. This interaction usually involves changes in gene transcription (trans-activation) by interacting with co-activator molecules. As the role of receptors is pivotal, two GR (GR1 and GR2) and also one mineralocorticoid receptor (MR) have been cloned and sequenced in several teleost species (Bury et al., 2003; Greenwood et al., 2003; Stolte et al., 2008a). In zebrafish (*Danio rerio*) a splice variant for GR similar than that of mammals has been described (Alsop and Vijayan, 2009, 2008). Interestingly, the circulating level of aldosterone is extremely low in fish and cortisol palliates this situation binding to MRs and playing a principal role, for example, in the acclimation of teleost to seawater environment (Mancera et al., 2002; McCormick, 2001; Prunet et al., 2006; Takahashi and Sakamoto, 2013). Therefore, in teleosts cortisol plays both glucocorticoid and mineralocorticoid functions through GRs and MRs, respectively (McCormick and Bradshaw, 2006).

Adrenaline is the main catecholamine product of the activation of SAM axis after sympathetic innervation of the head kidney chromaffin cells. Adrenaline modulates cardiovascular and respiratory function in order to rapidly mobilize the available energy reservoirs and to maintain an adequate oxygen level to satisfy the increased energy demand that the stress response implies (Reid et al., 1998). There is evidence of the β-adrenoreceptor existence which recognises adrenaline and mediates its effects (Chadzinska et al., 2012; Fabbri et al., 1998; Jozefowski and Plytycz, 1998). Thus, cortisol, ACTH and adrenaline are currently present in the head kidney after an stress episode.

Cytokines are key regulators of the immune response which are produced mainly at the site of entry of pathogens and regulate the activation of resident immune cells. Although a powerful inflammatory response is crucial to overcome an infection, this might be a double edged sword bringing damage to the host tissue, hence the provoked pro-inflammatory response has to be rapidly regulated (Chadzinska et al., 2008; Wang and Secombes, 2013). Among cytokines, IL-1β is an endogenous pyrogen (very responsive because of the fast expression), produced and released at the early stage response to infections, lesions and stress (Duque and Descoteaux, 2014). In fact, this cytokine is an initiator of pro-inflammatory response with key roles on the stimulation of pro-inflammatory mediators, such as other cytokines and prostaglandins in macrophages, and activation of lymphocytes (Alvarez-Pellitero, 2008; Dinarello, 2009). IL-1β can activate the expression of IL-6, a pleiotropic cytokine which has both pro- and anti-inflammatory functions, promoting differentiation of B-cells into plasma cells and activating cytotoxic T cells (Duque and Descoteaux, 2014). Another relevant actor in the pro-inflammatory response is TNF-α, which is able to exert its effect in many organs and induces IL-6 together with IL-β (Baud and Karin, 2001). This cytokine can stimulate the acute phase of immune response and is one of the first to be released in response to pathogens as well as after stress episodes (Teles et al., 2011). The pro-inflammatory cascade is strictly regulated by anti-inflammatory mediators, IL-10 and TGF-β1, responsible for maintaining the response under control to avoid tissue damage (Cools et al., 2007; Hu et al., 2008). IL-10 is produced by activated macrophages, B cells and T cells (Mosser and Edwards, 2008), while TGF-β1 has been described mostly associated with IL-10 as an immunosuppressive cytokine (Cools et al., 2007) regulating the immune response by blocking the activation of lymphocytes and monocyte-derived phagocytes and promoting tissue repair after a local inflammatory response (Li et al., 2009). Thus, in this study, we have analysed the hormone effect over some of the main cytokines involved in pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10 and TGF-β1) response.

The relationship between immune and neuroendocrine system is bidirectional because hormones affect immune cells but cytokines can also modulate HPI function (Calcagni and Elenkov, 2006). Although the neuroendocrine and immune systems were initially considered to act independently, it is now recognized that an extensive communication network controls an orchestrated neuroendocrine-immune interaction (Engelsma et al., 2002). Thus, it is not surprising that most of the immune cells such as lymphocytes, monocytes, macrophages and granulocytes express receptors for many neuroendocrine products and their expression is regulated after hormone secretion (Bury et al., 2003; Duque and Descoteaux, 2014). In previous results obtained in our group the effects of adrenaline, adrenocorticotropic hormone (ACTH) and cortisol on the expression of pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (TGF-β1) cytokines was evaluated in sea bream head kidney cells, showing a down-regulation in the expression of these cytokines after 2 h of treatment (Castillo et al., 2009). However, there is no evidence on whether antagonists for specific stress hormones receptors may revert the cytokine gene expression regulation observed after hormone administration. Therefore, the aim of this study was to assess the in vitro effects of the main stress hormones alone or in combination with their antagonist receptor on the gene expression of key pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β) in head kidney primary cell culture. We used spironolactone and mifepristone to antagonize GR and ACTH receptors (Alderman et al., 2012) and propranolol and phentolamine as β-adrenoreceptor and α-adrenoreceptor antagonists, respectively. Two fish species (rainbow trout and gilthead sea bream) were analysed in order to determine whether fish from different characteristics such as water environment or genetic background, respond similarly to the same experimental conditions. This is the first study to assess the effect of antagonist receptors over stress hormones regulation of immune-related genes.

2. Material and methods

2.1. Animals

Oncorhynchus mykiss of body weight of 120–140 g were obtained from a local fish farm (Piscifactoria Andres, St Privat d'en Bas, Spain). *Sparus aurata* with body weight of 60–70 g were obtained from AQUACULTURA ELS ALFACS,S.L. (Tarragona). Fish were transferred to the Universitat Autonoma de Barcelona (UAB) fish facility (AQUAB) to acclimatize them to laboratory conditions. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2. Head kidney primary cell culture (HKPCC) preparation

Rainbow trout (n = 6) and gilthead sea bream (n = 6) head kidneys were isolated from euthanized fish by overdose of MS-222 (Sigma). Head kidney was isolated and immediately placed in DMEM – high glucose (Sigma) and kept in ice. Tissue was homogenized through a 100 μm nylon cell strainer (Falcon). HKPCC was prepared in DMEM – high glucose (Sigma) at concentration of 2×10^6 cells/ml. The head kidney cells (1 ml) were left undisturbed in an incubator under optimal culture temperature conditions (16 °C for rainbow trout; 18 °C for sea bream, and 5% CO_2) for 2 h to settle in a 24-well culture plate (Jet Biofil) before adding