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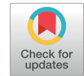
Cortisol and melatonin in the cutaneous stress response system of fish



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A B S T R A C T

The stress hormone cortisol, together with antioxidants, melatonin (Mel) and its biologically active metabolites, 5-methoxykynuramines, including AFMK, set up a local stress response system in mammalian skin. Our *in vitro* study of the European flounder (*Platichthys flesus*) was designed to examine whether Mel and AFMK would respond to cortisol while a glucocorticoid is added to the incubation medium. The concentrations of cortisol in the incubation medium mimic plasma cortisol levels seen in fish exposed to different types of stresses such as handling, confinement, high density, food-deprivation or air-exposure. We measured Mel and AFMK in skin explants and culture media using high-performance liquid chromatography (HPLC) with fluorescence detection. We also analysed melanosome response (dispersion/aggregation) in the explants subjected to the different treatments. Cortisol stimulated the release of Mel and AFMK from skin explants in a dose-dependent manner. Melanosome dispersion and a darkening of the skin explants were observed after incubation with cortisol. This study is the first to demonstrate the interrelationship between cortisol and Mel/AFMK in fish skin. Our data strongly suggest that the cutaneous stress response system (CSRS) is present in fish. The question remains whether Mel, AFMK or cortisol are synthetized locally in fish skin and/or transported by the bloodstream. The presence of the CSRS should be taken into account during elaboration of new indicators of fish welfare both in aquaculture and in the wild.

1. Introduction

The skin of vertebrates is an organ of protection and sensation. It acts as a biological barrier defending the organism against harmful environmental factors, such as mechanical impacts, variations in pressure and temperature, radiation, chemicals, microorganisms, and external parasites. Moreover, the skin contains an extensive network of nerve cells to detect changes in the environment and relay information to the central nervous system. It has been proposed by Slominski and colleagues, as early as 1995, that the human skin has a local mechanism of response to stress which is an equivalent to the hypothalamic–pituitary–adrenal axis (HPA) (Slominski et al., 1995). At that time, they introduced the term “skin stress response system”. Recent studies of mammalian skin cell biology have revealed many unexpected functions of the skin (Watt, 2014). There is substantial evidence that many hormones and other biologically active molecules are synthesized in specialized cells of the epidermis and dermis or released by adjacent neurons in response to suitable stimuli. For instance, mammalian skin is a source of stress hormones related to HPA, such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol, as well as being a site of melatonin (Mel) synthesis (Slominski et al., 1996; Slominski and Wortsman, 2000; Slominski et al., 2008, 2013; Acuña-Castroviejo et al., 2014). Melatonin, beyond its activity in circadian rhythm, is a highly sensitive and effective scavenger of hydroxyl and peroxyl radicals (Hardeland, 2005), and can stimulate the activity of antioxidative enzymes (Dzięgiel et al., 2003). Therefore, significant amounts of Mel are found in organs such as the skin, which are exposed to unfavourable environmental conditions (Tan et al., 2007). Because Mel metabolites such as N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK) are also potent scavengers, an inactivation of free radicals in cells is performed *via* the antioxidative cascade: Mel → AFMK → AMK (Hardeland, 2008; Galano et al., 2013). The efficiency of Mel, AFMK and AMK for scavenging depends on the polarity of the analytical medium and the type of free radical with which they are reacting (Hardeland et al., 2009; Galano et al., 2013).

In mammals, the cells synthesizing the hormones together with the nerve endings constitute the cutaneous neuroendocrine system which can respond to changes in the environment, and Mel and its derivatives, CRH, ACTH, and cortisol, are interconnected parts of this system (Slominski et al., 2005, 2012). It is also well established that multiple glucocorticoid receptors (GRs) are potent modulators of cutaneous

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homeostasis (Schmuth et al., 2007). Current evidence suggests that fish skin also has a stress response system. Firstly, cortisol, the main stress hormone in vertebrates including fish (Wendelaar Bonga, 1997), and Mel, an important antioxidant, are both present in fish skin (Bertotto et al., 2010; Kulczykowska et al., 2017). There is also evidence for GR localization in epidermal cells in the skin of zebrafish (*Danio rerio*) (Cruz et al., 2013). Secondly, the interactions between cortisol or stress exposure, and Mel production or its plasma levels have been shown in rainbow trout (*Oncorhynchus mykiss*), Mozambique tilapia (*Oreochromis mossambicus*), and gilthead sea bream (*Sparus aurata*) (Kulczykowska, 2001; Larson et al., 2004; Mancera et al., 2008, 2009; Nikaido et al., 2010; López-Patiño et al., 2014). Moreover, in several fish species, administration of cortisol or exposure to stress induces stress-related changes in the skin such as melanosome dispersion (Iger et al., 1995; Höglund et al., 2000; Ruane et al., 2005).

The purpose of this *in vitro* experiment with European flounder (*Platichthys flesus*) skin explants was to find out if Mel and its biologically active metabolites, AFMK and AMK, respond to cortisol while the glucocorticoid is added into the incubation medium. We investigated the release of Mel, AFMK, and AMK from the explants after incubation with different concentrations of cortisol mimicking stress conditions in fish. We also analysed the response of melanosomes while the explants are exposed to different treatments in order to validate the physiological relevance of this *in vitro* model. We aimed to answer the question whether cortisol and Mel, with its metabolites, are functionally interconnected to set up the cutaneous stress response system (CSRS) in fish. As yet, to the best of the authors' knowledge, the CSRS has not been studied in non-mammalian vertebrates.

2. Materials and methods

2.1. Experimental fish

Adult European flounders (*Platichthys flesus*) of both sexes were caught outside the spawning season in the Gulf of Gdańsk (southern Baltic Sea). The fish (n = 18) were maintained at a temperature of 10 °C in water of 8 ppt salinity with a 12L:12D photoperiod in aerated aquaria at the Institute of Oceanology PAN (IO PAN Sopot, Poland), where all experiments were carried out. The fish were acclimatized for one week before experimentation and fed frozen mussels *ad libitum* once a day, at noon. The randomly selected fish were anaesthetized by immersion in 0.5% (v/v) 2-phenoxyethanol (Sigma–Aldrich, USA) solution in water, their spinal cords were sectioned and skin samples collected. Skin samples were taken from 10 fish to establish cortisol concentrations for *in vitro* study; samples from the 8 other fish were either used in *in vitro* study or stored at −70 °C until the analyses of Mel, AFMK and AMK concentrations were conducted. The *in vitro* experiment was repeated 6 times.

All experiments complied with EC Directive 2010/63/EU for animal experiments and with the guidelines of the Local Ethics Committee on Animal Experimentation.

2.2. Analysis of cortisol

Skin samples (10 mm × 10 mm) were weighed, fragmented using a scalpel blade and homogenized in 0.05 M phosphate buffer (pH 7.4) containing 0.01% Thimerosal (Sigma-Aldrich, USA) using an ULTRA-TURRAX homogenizer (IKA, USA). The extracts were centrifuged at 15000*g* for 20 min at 4 °C, and then supernatants were decanted and stored at −70 °C prior to the analysis of cortisol levels. Cortisol levels were determined using a solid phase enzyme-linked immunosorbent assay (ELISA) kit (DRG, Germany). A standard curve was prepared using six standard dilutions of cortisol: 20, 50, 100, 200, 400 and 800 ng/mL. The assay was conducted in microplates according to the ELISA kit manufacturer's instructions, and based on the principle of competitive binding: cortisol in standards and samples competed with cortisol conjugated to horseradish peroxidase for the antibody binding sites in the microtiter wells. Microplates were incubated for 60 min at room temperature, and unbound components were then washed away with buffer using a HydroFlex strip-washer (Tecan, Austria). Bound cortisol enzyme conjugate was measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). The reaction was carried out at room temperature for 15 min, and stopped by the addition of 100 μL of 0.5 M H_2SO_4. The absorbance at 450 nm was read using a Sunrise Absorbance Reader (Tecan, Austria) within 10 min after stopping the reaction. All samples were assayed in duplicate. The detection limit of the assay was 14.77 ng/mL. The intra-assay coefficient of variation was 0.87%. The inter-assay variation was not determined because all samples were measured in the same assay. The mean cortisol concentration in the skin was 24.99 ± 0.40 ng/g (number of fish = 10).

2.3. The dynamic culture of skin explants

Skin explants (10 mm × 10 mm) were put in sterile Petri dishes and placed in the incubator with shaking (45 cycles/min) (Heidolph, Germany) at 10 °C and aerated using atmospheric air at a pressure of 127.51 mm Hg. The explants were washed twice with Leibovitz's L-15 Medium at pH 7.4 (L-15; Sigma-Aldrich, USA) for 10 min, rinsed in L-15 Medium supplemented with Penicillin (500 UI/mL), Streptomycin (0.5 mg/mL) and Neomycin (1 mg/mL) (Sigma-Aldrich, USA) for 5 min, and in L-15 Medium for 10 min in accordance with the method applied by Sugimoto et al. (2000). All samples were then incubated in sterile 6-well plates (CELLSTAR, Greiner Bio-One, Germany) with inserts (Cell Crown, Sigma-Aldrich, USA) under the same conditions as at the beginning of the experiment (shaking: 45 cycles/min at 10 °C and aeration pressure: 127.51 mm Hg). The explants were put on the Nylon Net Filter 20 μm (NY2002500; Merck Millipore, Germany) and incubated in L-15 Medium supplemented with L-glutamine, 10 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) and 1 mM $NaHCO_3$ (Sigma-Aldrich, USA) at pH 7.4. Each culture plate contained two control wells (medium without cortisol) and four wells with different concentrations of cortisol (50, 150, 250 or 1250 ng/mL). After a 60-min stabilization period (data not shown), all the incubations were carried out in 30-min cycles with medium replacement. During the first 30-min incubation period, the initial Mel, AFMK and AMK release was determined. The explants were then incubated with four different concentrations of cortisol (50, 150, 250, 1250 ng/mL). In the last three periods, explants were incubated in medium without cortisol. The cortisol concentrations in the incubation medium were 2, 6, and 10 times higher than the cortisol concentration measured in the skin of flounders not subjected to stress (24.99 ± 0.40 ng/g). All procedures were carried out under red light in a laminar air flow cabinet (NUAIRE Biological Safety Cabinet Class II, USA). All collected media were centrifuged at 4 °C at 3000*g* for 10 min and supernatants were stored at −70 °C prior to analysis.

2.4. Analysis of melanosome aggregation and dispersion in skin explants

The effect of melanosome dispersion/aggregation was studied in dorsal skin explants after 60 min of incubation in medium without cortisol, with four different doses of cortisol and a high concentration of K^+ (60 mM). Skin explants (n = 18) were fixed in 4% buffered formalin and mounted with Eukitt (Sigma-Aldrich, USA). The diameter of 40 randomly selected melanophores was measured for each explant. The analysis was taken under Leica M205C Microscope with planochromatic objective connected with Leica DFC450 digital color camera, using Leica Application Suite (LAS) 4 software enhanced with Interactive Measurements module (Leica Microsystems GmbH, Germany). The melanophore index (MI) was used to estimate the degree of pigment dispersal in fish, in accordance with the method applied by Aspengren et al. (2003). Using this method, a fully dispersed

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