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A role for melatonin in maintaining the pro- and anti-inflammatory balance by influencing leukocyte migration and apoptosis in carp

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ABSTRACT

Melatonin is responsible for the synchronization of many physiological processes, including the immune response. Here we focus on the expression of melatonin MT1 receptors in/on leukocytes, and on the effects of melatonin administration on the inflammatory processes of carp.

For the first time, we showed that fish leukocytes express MT1 receptors, implicating direct responsiveness to melatonin stimulation. Moreover, both *in vitro* and *in vivo*, melatonin modulated the immune response. The most potent effects of melatonin concerned the regulation of leukocyte migration. Melatonin reduced chemotaxis of leukocytes towards CXC chemokines *in vitro*. *In vivo,* during zymosan induced peritonitis, i.p. administration of melatonin reduced the number of neutrophils. This correlated with a melatonin-induced decrease of gene expression of the CXCa chemokine. Moreover, melatonin induced a decrease of the respiratory burst in inflammatory leukocytes.

Although these data do suggest a potent anti-inflammatory function for this hormone, melatonin-induced inhibition of leukocyte apoptosis clearly indicates towards a dual function.

These results show that also in carp, melatonin performs a pleiotropic and extra-pineal function that is important in maintaining the delicate pro- and anti-inflammatory balance during infection. They furthermore demonstrate that neuroendocrine–immune interaction via melatonin is evolutionary conserved.

1. Introduction

The “hormone of darkness” (Reiter, 1991), melatonin (*N*-acethyl-5-methoxytryptamine), is a crucial mediator responsible for synchronization of many physiological processes in almost all organisms and its molecular structure is highly conserved (Tan et al., 2010). Melatonin is produced during the dark phase and therefore is mainly involved in the regulation of day/night rhythms and/or seasonal activity (Arendt and Skene, 2005). Although in vertebrates the pineal gland is the main source of melatonin, it can also be produced in the gastrointestinal tract, eyes, skin, brain, gonads as well as in the lymphoid organs e.g. in the thymus and bone marrow (Srinivasan et al., 2008; Tan et al., 2007). Leukocytes posses all key enzymes necessary for melatonin synthesis (Sainz et al., 2003). In contrast to the chronobiotic effects that are mediated by melatonin from pineal origin, hormone production by other cells including leukocytes, is independent of the light/dark cycle and exerts nonchronobiotic autocrine or paracrine effects (Cecon and Markus, 2011; Calvo et al., 2013; Tan et al., 2007).

Melatonin is involved in the bidirectional interaction of the neuroendocrine and immune systems (Skwarlo-Sonta, 2002) and melatonin receptors MT1/MT2 are expressed on leukocytes (Pozo et al., 2004). Melatonin influences the development of the thymus, spleen and bursa (Carrillo-Vico et al., 2005; Skwarlo-Sonta, 1999). Furthermore, both *in vitro* and *in vivo* it regulates the innate and adaptive immune response (García-Mauriño et al., 1999). During inflammation in mammals it affects the vascular permeability, the recruitment of leukocytes (Lotufo et al., 2006) and the expression of pro- and anti-inflammatory mediators (García-Mauriño et al., 1999). Melatonin moreover up-regulates the synthesis of many cytokines e.g. interleukins: IL-1, IL-2, IL-6, IL -12, interferon gamma (IFN-γ) and tumor necrosis factor alfa (TNF-α) (Garcia-Maurino et al., 1997, 1999; Barjavel et al., 1998). The positive

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effect on IFN-γ and IL-6 synthesis indicates that melatonin acts on T-helper cells in favor of the cellular Th1 response (Carrillo-Vico et al., 2005). This effect is even further boosted by melatonin-induced increase of IL-12 production (García-Mauriño et al., 1999). In contrast, IL-4, the cytokine specific for the Th2 response is not stimulated by melatonin (García-Mauriño et al., 1999), while IL-10 is even down-regulated (Kühlwein and Irwin, 2001). Also antibody production may be modulated by melatonin (Cernysiov et al., 2010). In birds, pinealectomy leads to a depression of humoral and cellular immunity which can be reversed by melatonin replacement (Moore and Siopes, 2002). Interestingly, in mammals, cytokines derived from the immune system may act as regulator for melatonin synthesis in the pineal gland (Markus et al., 2007). The pro-inflammatory TNF-α, produced at the site of inflammation and subsequently transported to the pineal gland, inhibits melatonin synthesis via activation of the NF-κB pathway (Fernandes et al., 2006).

In mammals, melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), act as scavengers for free radicals (Sainz et al., 2003) and can up-regulate several antioxidant enzymes like Mn-SOD, CuZn-SOD and GPx (Fischer et al., 2008). Melatonin exerts its effects via membrane and nuclear receptors, as well as via receptor independent actions (Fischer et al., 2008). Melatonin induced inhibition of inducible nitric oxide synthase (iNOS) gene expression and its scavenging of reactive oxygen (ROS) and nitrogen (RNS) species, was hypothesized to be a crucial anti-inflammatory (Mayo et al., 2005), oncostatic (Pandi-Perumal et al., 2006) and anti-aging factor (Cardinali et al., 2008). This inhibition of intracellular ROS may be an important anti-apoptotic factor by preventing the activation of caspase-9, caspase-3 and DNA damage (Espino et al., 2011). The anti-apoptotic action of melatonin is therefore probably mediated via down-regulation of the mitochondrial apoptotic pathway (Hoijman et al., 2004). Interestingly the antioxidant action of melatonin in leukocyte apoptosis is independent of plasma membrane MT1/MT2 receptor stimulation (Espino et al., 2011).

In fish, melatonin plays a role in reproduction, growth and behavior (Falcón et al., 2009; Conde-Sieira et al., 2012). Radioligand binding studies for fish cells allowed the identification of three high affinity melatonin receptor subtypes, all belonging to the GPCR family: MT1, MT2 and Mel1c (Falcón et al., 2007). Full length cloning of melatonin receptors has been performed for trout (MT1), rabbitfish (MT1, Mel1c), seabass (MT1, MT2, Mel1c), pike (MT2) (Sauzet et al., 2008) and Senegalese sole (MT1, MT2 Mel1c) (Confente et al., 2010), while full length sequences predicted from the genome analysis of several other fish species are available from the databases. Functional studies have only been performed for the fish MT2 receptor, which appears negatively coupled to the cAMP pathway (Falcón et al., 2010).

Very little is known about involvement of melatonin in the regulation of the immune response in teleosts. In rainbow trout (*Oncorhynchus mykiss* L.), seasonal changes in melatonin synthesis correlate with changes in total white blood cell counts and lysozyme activity, but not with synthesis of reactive oxygen species (ROS) during the respiratory burst (Morgan et al., 2008). The humoral innate immune system of seabream (*Sparus aurata* L.) and sea bass (*Dicentrarchus labrax* L.), exposed to a constant light–dark photoperiod, showed pineal dependency of the circadian rhythms of the humoral responses (Esteban et al., 2006). In gilthead seabream melatonin *in vivo* enhances the activity of leukocytes: activity of peroxidase, phagocytosis, ROS production and expression of immune-related genes (IL-1β, MHC, Mx) and lymphocyte markers (Cuesta et al., 2008). *In vitro* incubation of seabream and sea bass leucocytes with low doses of melatonin did not change their activity, whereas very high (pharmacological) doses inhibited the peroxidase activity and increased ROS synthesis (Cuesta et al., 2007).

The present study focuses on the innate immune response of carp and analyses the expression of MT1 melatonin receptors on leukocytes as well as the effect of *in vitro* and *in vivo* administration of its ligand – melatonin. We show expression of melatonin membrane receptors on leukocytes and show that melatonin affects the inflammatory response through altered cell migration and apoptosis. Melatonin thus executes pleiotropic and extra-pineal functions that may be crucial to preserve the subtle pro- and anti-inflammatory equilibrium during infection.

2. Materials and methods

2.1. Animals

Young individuals of common carp (60–70 g, *Cyprinus carpio* L.) (body weight), from the Institute of Ichthyobiology and Aquaculture, Polish Academy of Science, Golysz, Poland were reared at 20 °C in recirculating tap water at the aquatic facility of the Institute of Zoology, Jagiellonian University in Krakow. Fish were fed dry food pellets (Trouvit, Nutreco) at daily maintenance rate of 1% of their estimated body weight. The tanks were equally positioned to avoid unnecessary additional interference and stress. Animals were kept in a 12 h:12 h of dark/light cycle. All injections and samplings were carefully performed by the same person and at the same time of the day to avoid differences in handling. Animals were anaesthetized with tricaine methane sulphonate (TMS; Sigma–Aldrich, St. Louis, MO; 0.2 g/l) buffered with NaHCO3 (POCH, Gliwice, Poland; 0.4 g/l).

All experiments were conducted according to license no. 23/2012 from the local ethical committee.

2.2. Organ and cell isolation

Organs (head kidney, spleen and thymus) were carefully removed, snap frozen in solid CO_2 or liquid N_2 and stored at −80 °C. Peripheral blood leukocytes were isolated as described previously by Verburg-van Kemenade et al. (1995).

Head kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with carp RPMI (cRPMI) (RPMI 1640, Invitrogen, Carlsbad, CA, adjusted to carp osmolarity of 270 mOsm/kg with distilled water) containing 10 IU/ml heparin (Leo Pharmaceutical Products Ltd, Weesp, the Netherlands) and washed once. This cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.020, 1.060, 1.070 and 1.083 g/cm^3) and centrifuged for 30 min at 800 g with the brake disengaged.

Cells at 1.060, 1.070 and 1.083 g/cm^3 fractions (Kemenade et al., 1994) were collected, washed and further used either to study the MT1 melatonin receptor expression or the *in vitro* effects of melatonin on leukocyte activity.

2.3. Expression of the MT1 receptor

2.3.1. Immunocytochemical studies

Cytospins containing 1×10^6 head kidney lymphocytes, monocytes/macrophages or neutrophillic granulocytes were prepared on slides by centrifugation (5 min, 447 g; Hettich Universal Tuttlingen, Germany), immediately fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO) and stored at −20 °C until analysis.

Before staining, cytospins were rehydrated, washed in 0.1 M pH 7.3 phosphate-buffered saline (PBS) and phosphate-buffered saline