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Research Article

The Effects of Melatonin Hormone on Carbonic Anhydrase Enzyme Activity in Rainbow Trout (*Oncorhynchus mykiss*) Erythrocytes In Vitro and In Vivo

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Abstract: The effects of melatonin hormone on rainbow trout (*Oncorhynchus mykiss*) erythrocyte carbonic anhydrase enzyme purified by using Sepharose 4B-L tyrosine-sulfanylamide affinity gel chromatography were investigated in vitro and in vivo. Carbonic anhydrase from *Oncorhynchus mykiss* erythrocytes was purified with a specific activity of ~400 EU/mg protein and ~200-fold in a yield of ~20. Melatonin did not inhibit carbonic anhydrase enzyme in vitro. Carbonic anhydrase activity of the control group was determined as 1597.7 ± 429.0 EU/g hemoglobin in vivo. Carbonic anhydrase activities were measured at 1, 3 and 5 h by following melatonin injection. The corresponding mean activities of melatonin were 1406.4 ± 372.1, 1346.3 ± 477.4 and 783.8 ± 494.3 EU/g hemoglobin respectively. *Oncorhynchus mykiss* erythrocyte carbonic anhydrase was inhibited significantly by melatonin hormone 5 h after injection in vivo ($P < 0.05$). Data from the present study suggest that excessive usage of this hormone should be avoided in fish reproduction.

Key Words: Carbonic anhydrase, erythrocyte, melatonin, *Oncorhynchus mykiss*, in vitro, in vivo

Melatonin Hormonunun Gökkuşağı Alabalığındaki (*Oncorhynchus mykiss*) Eritrosit Karbonik Anhidraz Enzimi Üzerine In Vitro ve In Vivo Etkileri

Özet: Bu çalışmada, Sepharose 4B-L tirozin-sülfanilamid afinite jel kromatografisi ile saflaştırılan gökkuşağı alabalığı (*Oncorhynchus mykiss*) eritrositlerindeki karbonik anhidraz enzimi üzerine melatonin hormonunun etkileri araştırılmıştır. CA enzimi *Oncorhynchus mykiss* eritrositlerinden ~20 verim ile ~200 kat saflaştırılmıştır. Enzimin spesifik aktivitesi ise ~ 400 EU/mg protein olarak hesaplanmıştır. In vitro çalışmalarda melatonin hormonunun karbonik anhidraz enzimini inhibe etmediği tespit edilmiştir. In vivo çalışmalarda, kontrol grubu balıklarda karbonik anhidraz enzim aktivitesi 1597,7 ± 429,0 EU/g hemoglobin; melatonin enjeksiyonundan sonra 1., 3. ve 5. saatlerdeki karbonik anhidraz aktiviteleri ise sırasıyla 1406,4 ± 372,1, 1346,3 ± 477,4 ve 783,8 ± 494,3 EU/g hemoglobin olarak belirlenmiştir. *Oncorhynchus mykiss* eritrosit karbonik anhidraz enziminin melatonin hormonu tarafından enjeksiyonu takiben 5. saatte önemli seviyede ($P < 0,05$) inhibe edildiği tespit edilmiştir. Çalışmadan elde edilen veriler göz önüne alındığında melatonin hormonunun üreme dönemindeki balıklarda aşırı miktarlarda kullanılmaması gerektiği önerilebilir.

Anahtar Sözcükler: Karbonik anhidraz, eritrosit, melatonin, *Oncorhynchus mykiss*, in vitro, in vivo

Introduction

Melatonin is primarily produced by the photoreceptor cells, which act as a photoneuroendocrine transducer and secrete melatonin into the blood, in the pineal organ (1,2). It is involved in many physiological processes, such as seasonal reproduction, activity rhythms, and sleep/wake cycles (3), in osmoregulation and/or stress adaptation in seawater (4). Moreover, it is considered as a powerful free radical scavenger and likely to be a general promoter of anti-oxidative mechanisms and a

potential antioxidant in vitro and in vivo (5). Some of its interactions with other hormones such as cortisol and arginine vasotocin (hormones closely associated with electrolyte balance) have been reported in teleosts (6,7). It was also reported that melatonin can alter the activities of enzymes that improve the total antioxidative defense capacity of the organism, e.g., superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose 6-phosphate dehydrogenase and nitric oxide synthase (8).

Carbonic anhydrase (CA) is known as a very important enzyme regulating CO_2 levels in living organisms. Although fourteen different CA isozymes were described so far in higher vertebrates, their only known physiological function is to facilitate the interconversion of CO_2 to HCO_3^- and H^+ (9). In fish, it exhibits a fundamental role in a number of physiological processes such as pH control and gas balance, calcification, osmoregulation, ionoregulation and clearance of the waste products from nitrogenous metabolism (10). Raisanen et al. (11) stated that CA III functions as an oxyradical scavenger and thus protects cells from oxidative stress, which has many adverse effects, including lipid peroxidation, protein oxidation and interference with cellular homoeostasis, which can lead to cell death and pathological injury (12).

Although the influence of melatonin administration on fish reproduction has been extensively investigated (13-15), studies related to the effects of this application on enzyme inhibition or activation are rare. Since CA and melatonin hormone are important for similar physiological functions (osmoregulation, ionoregulation, oxyradical scavenger, etc.) as mentioned above and *O. mykiss* is among the widely cultured fish in the world, being familiar with the possible effects of this hormone on activity of CA enzyme would be useful for further studies on this ubiquitous enzyme in the physiological and biochemical fields. Therefore, the present study aimed to determine the in vitro and in vivo effects of melatonin hormone on enzyme activities of CA in *O. mykiss* erythrocyte.

Materials and Methods

Fish husbandry and maintenance

Individuals of *O. mykiss* were held and bred in storage tanks in Central Laboratory at Aquarium Fish Rearing Facility of Fisheries Department of Agricultural Faculty at Atatürk University. The tanks were subjected to a natural photoperiod and supplied with constantly running aerated and dechlorinated city water. The average temperature, dissolved oxygen, pH and total hardness of rearing water were 9 ± 2 °C, 8-9 ppm, 7.8 and 102 mg as $CaCO_3$ respectively during the tests. Fish were fed a commercial trout diet with 51% protein, 17.5% fat, 93.93% dry matter and 9.3% ash twice a day at 1% of their live body weight.

Purification of CA from *O. mykiss* erythrocytes by affinity chromatography

Thirty fish (250 ± 10 g, mean ± SD) were used for the blood samples. In the laboratory, the fish were first anaesthetized by using triciaine methanesulfonate (MS-222); then blood samples were taken from their caudal veins by heparinized syringes (16). The blood samples were centrifuged at 1500 rpm for 15 minutes and the plasma and buffy coat were removed. The erythrocyte cells were isolated, washed twice with 0.9% NaCl solution and hemolyzed with 1.5 volumes of ice-cold distilled water. For the removal of the ghost and intact cells, hemolysate was centrifuged at 12,000 rpm for 45 minutes. The hemolysate pH was adjusted to 8.7 with the solid Tris and then it was applied to the prepared Sepharose 4B-L-tyrosine-sulfanylamide affinity column. The affinity gel was washed with the solution of 25 mM Tris-HCl / 22 mM Na_2SO_4 (pH 8.7) and *O. mykiss* carbonic anhydrase enzyme was eluted with the solution of 0.1 M $NaCH_3COO$/ 0.5 M $NaClO_4$ (pH 5.6). Finally, the eluted enzyme solution was dialyzed against 0.01 M potassium phosphate/ 0.1 M KCl/ 5 mM 2-mercaptoethanol (pH 7.4). All the progresses mentioned above were carried out at 4 °C (17).

Measurement of CA activity

CO_2-hydratase activity of carbonic anhydrase enzyme in *O. mykiss* erythrocytes was assayed colorimetrically using the method of Wilbur and Anderson (18). CO_2-Hydratase activity as an enzyme unit (EU) was calculated by using the equation $[(t_0-t_c)/t_c]$, where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Protein determination

During the purification steps, protein levels were determined spectrophotometrically according to the Bradford method (19), using bovine serum albumin as the standard. Amount of protein in column fractions was observed via absorbance variations at 280 nm.

SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed after purification of the erythrocyte CA. The running and stacking gel was prepared according to the method of Laemmli (20) with acrylamide concentrations of 10% and 3%. Both gels consisted of 0.1% SDS. Twenty-mg samples were applied to the electrophoresis medium. The gel was later stained in a solution of 10% acetic acid and 50% methanol containing 0.1% Coomassie Brilliant Blue R-250. Afterwards, it was destained with several changes of the same solvents not containing Coomassie Brilliant Blue R-250.

In vitro experiment

Melatonin hormone (0.01, 0.02, 0.04, 0.06 and 1 mM) were added to the medium of enzyme activity determination (total volume: 4.2 ml) at five different concentrations. According to Çiftçi et al. (21) 0.06 mM was regarded as pharmacological dose and 1 mM as an overdose; especially small doses from 0.06 mM were tested to see the enzyme activity. CA activities with melatonin hormone were assayed by following the hydration of CO_2 and repeated three times. Activity % values of CA enzyme for five different concentrations of melatonin hormone were drawn by using regression analysis graphs on a computer. CA activity without any compound was regarded as 100% activity. For the compounds having an inhibition effect, the inhibitor concentrations causing up to 50% inhibition (I_{50} values) were determined from the graphs.

In vivo experiment

Twenty rainbow trout were sorted into a 785-l circular fiberglass tank and held for a week for acclimation; 0.5 ml blood samples were taken from 5 randomly selected fish (replicate) to determine initial mean CA activities as a control group and placed into a heparinized vacutainer. Melatonin was dissolved in a small amount of ethanol and diluted with teleost saline (20 mg Na_2CO_3 /100 ml of 0.6% NaCl) until the desired final concentration were obtained. Melatonin solution (10 mg kg^{-1} fish) was injected into the remaining fish into the muscle around the dorsal fin (22). Starting from the injection time, the blood samples were taken from 5 fish of each group at 1, 3 and 5 hours. All blood samples were centrifuged at 3000 rpm, then the erythrocyte pellet was washed with 0.16 M KCl three times and the supernatant was discarded. One volume of erythrocyte pellet was hemolyzed in five volumes of ice-water and works on hemolysates were carried out at 4 °C. CA activity was assayed by following the hydration of CO_2.

Statistical analyses

The obtained data were subjected to one way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine significant difference among means at the $p = 0.05$ level.

Results

In the purification procedures, total volume of the hemolysate was 80 ml with 2240 mg total protein and a 1.9 EU/mg-protein specific activity. The yield was assumed as 100% at this stage. Following Sepharose 4B-L-tyrosine-sulfanylamide affinity gel chromatography, the total volume obtained was 15 ml with 2.13 mg total protein, a 15.2 EU/mg-protein specific activity and 11.3% yield. Finally following dialysis, the total volume was 15 ml with 2.13 mg total protein, a 422.5 EU/mg protein specific activity and 20.9% yield. After hemolysate preparation, Sepharose 4B-L-tyrosine-sulfanylamide affinity gel chromatography and dialysis, the enzyme was purified 222.4-fold. In order to assess the purity of the enzyme, SDS polyacrylamide gel electrophoresis was used and its electrophoretic pattern showed a single band (Figure 1).

Figure 1. SDS-polyacrylamide gel electrophoresis of CA purified by affinity gel. [(Lane 1: Standard proteins; (Rabbit muscle myosin 205,000 Da, α_2-Macroglobulin 180,000 Da, rabbit phosphorylase B 97,400 Da, bovine serum albumin 66,000 Da and bovine carbonic anhydrase 29,000 Da) Lane 2: *O. mykiss* erythrocyte CA; Lane 3: *O. mykiss* gill CA; Lane 4: *O. mykiss* lens CA)].

It was observed that melatonin did not inhibit the *O. mykiss* erythrocyte CA enzyme activity in vitro (Figure 2), but inhibited it in the in vivo experiment. CA activity of the control group was determined as 1597.7 ± 429.0 EU/g hemoglobin in vivo. Following melatonin injection, mean CA activities were 1406.4 ± 372.1, 1346.3 ± 477.4 and 783.8 ± 494.3 EU/g hemoglobin at 1, 3 and 5 h, respectively. The results showed that *O. mykiss* erythrocyte CA was inhibited significantly by melatonin hormone 5 h after injection in vivo ($P < 0.05$).

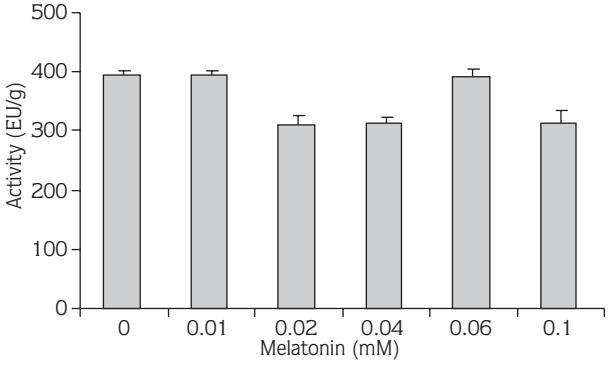

Figure 2. In vitro changes in activity (EU/g) of erythrocyte CA enzyme from *O. mykiss* in presence of 5 different melatonin hormone concentrations. (0.06 mM is regarded as a pharmalogical dose or maximum dose and 0.1 mM as an overdose). Values are expressed as mean ± SD (n = 3).

Discussion

It was reported that CA can be purified from many species of animals, plants, yeasts and bacteria (22). Different chromatography methods have been used to purify CA in fish (23). However, these methods such as sephadex G-75 gel filtration and DEAE Bio Gel anion exchange chromatography require considerable labor and take too much time. Therefore, Sepharose 4B-L tyrosine-sulfanylamide affinity chromatography was used in this study because it takes less time and permits the enzyme to be concentrated to a greater extent (17).

Purification with Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography resulted in a single 29 kDa molecular weight CA isozyme with high specific activity that was present in erythrocytes of *O. mykiss*. This result was similar to the findings (28.3 kDa) reported by Hall and Schraer (23).

Melatonin did not inhibit the CA enzyme activity in the in vitro experiment, but the in vivo experiment showed that there was a significant decrease in CA enzyme activity 5 h after melatonin injection. In contrast, it was reported that both in vitro and in vivo pharmacological levels of melatonin increased glucose-6-phosphate dehydrogenase (G6PD) enzyme activity in rat and human erythrocytes (21).

The fact that melatonin hormone had an inhibitory effect in vivo but non-inhibitory effect in vitro showed that it bound specific melatonin binding sites (24) and then led to inhibition on the CA enzyme. Alternatively melatonin can be metabolized and its metabolic products can cause inhibition by indirectly influencing the different biochemical pathways. For instance, Pinillos et al. (14) reported that the inhibitory effect of both melatonin and its agonist 2-iodomelatonin administered peripherally on food intake was mediated via luzindole-sensitive melatonin receptors in goldfish. It has been also reported that melatonin implantation can alter the timing of seawater adaptation and additionally, significantly alter the population structure of sibling in Atlantic salmon parr (25). Moreover, Gupta and Premabati (26) showed that melatonin was involved in the regulation of thyroid hormones in *Clarias gariepinus*. Additionally, Bornestaf et al. (13) found that high melatonin dose had an inhibitory effect on maturation pace under LD 24:0 in female three-spined stickleback. However, Amano et al. (15) reported that melatonin treatment had a stimulatory effect on the gonadosomatic index and pituitary gonadotropin (GTH) I contents in male masu salmon reared under LD 16:8 (lights on 04:00–20:00 h) and fed pellets sprayed with melatonin (0.5 mg melatonin/kg body weight/day).

From the result of the effects of the significant inhibition of CA enzyme activity, it can be argued that important physiological changes may occur in the body of the organisms as reported in the following studies. It was reported that CO_2 and NH_3 excretion was linked by the action of CA in trout white muscle (27). It has also been reported that respiratory acidosis in the blood of trout occurred by the inhibition of red cell CA activity (28).

A large increase in both arterial and muscle P-CO_2 indicating retention of CO_2 was reported with inhibition of red cell CA activity by acetazolamide infusion following exercise in trout (29). Moreover, it was reported that creatine phosphate resynthesis was delayed by CA inhibition in exercised white muscle of trout (30).

In conclusion, melatonin hormone inhibited the CA of erythrocyte in vivo. Therefore, it may demolish physiological events, i.e. preservation red cell intracellular

pH, ventilatory control and red cell fragility. For this reason, dosage, duration and methods of administration of melatonin should be further evaluated and excessive applications should be avoided.

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