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Effects of Melatonin on Carbonic Anhydrase from Human Erythrocytes *In Vitro* and from Rat Erythrocytes *In Vivo*

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The *in vitro* effects of melatonin (*N*-acetyl-5-methoxytryptamine) on human carbonic anhydrase isozymes (HCA-I and HCA-II) from human erythrocytes and *in vivo* effects on rat erythrocytes carbonic anhydrase (CA) were determined. Human erythrocyte carbonic anhydrase isozymes were purified by haemolysate preparation and Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography. The HCA-I enzyme, having a specific activity of 7337.5 EU/mg protein, was purified 843-fold with a yield of 60% and the HCA-II enzyme, having a specific activity of 17067 EU/mg protein, was purified 1962-fold with a yield of 22.7%. For *in vitro* experiments, the enzyme activity was minimal at 2×10^{-4} M melatonin concentration and increased above this concentration. Ten mg kg^{-1} melatonin was administered intraperitoneally and showed a stimulatory effect on the enzyme. Time-dependent *in vivo* studies were conducted for melatonin in Sprague–Dawley type rats. It was found that CA activity in the rat erythrocytes was decreased by the melatonin after 1 and 3 hours to 2500 ± 500.0 and 1875 ± 239.4 respectively which were statistically significant ($p < 0.05$) differences to the control (2660 ± 235.8). However, CA activity was restored to its normal level after 6 h (2666 ± 235.7) ($p > 0.05$) probably due to metabolism of the melatonin. The findings indicate that melatonin may be pharmacologically useful in some diseases.

Keywords: Melatonin; Carbonic anhydrase; Erythrocyte; Human and rat

INTRODUCTION

The pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine), an indoleamine, is the chief secretory product of the pineal gland. It is synthesized enzymatically from serotonin (5-hydroxytryptamine) by the sequential action of serotonin *N*-acetyltransferase and hydroxyindole-O-methyltransferase.[1] Melatonin is also produced in other organs and found in all body fluids after its release from the pineal. It is known that melatonin influence a variety of biological processes including circadian rhythms, neuroendocrine, cardiovascular and immune functions as well as thermoregulation.[2–4]

Carbonic anhydrase (CA, carbonate hydrolyase, EC 4.2.1.1) is a metalloenzyme. CAs are an important class of enzymes used to regulate CO_2 levels in living organisms by catalysing the reversible hydration of CO_2 to HCO_3^- and H^+.[5–7] Fourteen different carbonic anhydrase (CA) isozymes have been described up to now in higher vertebrates.[8–10] The only known physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3^-; therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis.[11] The enzyme has been extensively studied and purified from numerous sources including human erythrocytes, rat erythrocytes, rat saliva, *Plasmodium falciparium*, bovine bone and bovine leucocytes. This enzyme is widely distributed in most tissues such as eye, kidney and the central nervous system. CA isozymes activities in human erythrocytes have been shown to vary considerably under physiological conditions.[12–15] Moreover, changes in CA activity have been associated with metabolic diseases, such as diabetes mellitus and hypertension.[5,16] In a previous study, we determined the effects of gentamisin sulfate on human erythrocyte carbonic anhydrase *in vitro* and rat erythrocyte CA *in vivo*.[12]

In the present report the effects of melatonin, which had previously been shown to protect cellular components from free radical damage,[17–21] were

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investigated on carbonic anhydrase enzyme activity in *in vitro* human and in *in vivo* rat erythrocytes.

MATERIALS AND METHODS

Chemicals

Melatonin, Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. (Taufkirchen, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from E. Merck (Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Purification of Human Erythrocyte Carbonic Anhydrase Isozymes by Affinity Column Chromatography

Erythrocyte CA's were purified from fresh human blood obtained from the University Hospital Blood Centre, (10 ml). Following low-speed centrifugation (1500 rpm for 15 min) (MSE, MISTRAL 2000) and plasma and buffy coat were removed. The red cells were isolated, washed twice with 0.9% w/v NaCl and haemolysed with 1.5 volumes of ice-cold water. Ghost and intact cells were removed by high-speed centrifugation (20000 rpm for 30 min) (Heraeus Sepatech, Suprafuge 22) at 4°C and the haemolysate adjusted pH to 8.7 with solid Tris. The pH-adjusted haemolysate was then subjected to affinity chromatography [*Chromatography system:* chromatography column: 1.36 × 30 cm (Sigma Chemical Company), bed volume: 25 ml; peristaltic pump (Pharmacia Chemical Company), and fraction collector (AO Instrument Company, U.S.A.)] at 4°C for the purification of human erythrocyte carbonic anhydrase isozymes.[22]

The pH-adjusted human erythrocyte haemolysate (100 ml) was applied to the Sepharose 4B-L-tyrosine-sulfanylamide affinity column pre-equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (HCA-I and HCA-II) isozymes were eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), respectively (flow rate: 20 ml h^{-1}, fraction volume: 4 ml). During the human carbonic anhydrase isozymes purification procedures, the absorbance at 280 nm was used to monitor protein elution by affinity column chromatography.

Activity Determination

Carbonic anhydrase activity was assayed by following the hydration of CO_2 at room temperature according to the method described by Wilbur and Anderson.[23] 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

Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford's method, with bovine serum albumin as standard.[24]

SDS-polyacrylamide Gel Electrophoresis

Enzyme purity was controlled by SDS-polyacrylamide gel electrophoresis. This technique was performed according to the Laemmli's method using a vertical slab gel apparatus.[25] It was carried out in 10% and 4% acrylamide concentrations for running and stacking gel, respectively, containing 10% SDS. Gel was stained with Coomassie brilliant blue R-250 dye reagent overnight. The electrophoretic pattern was photographed (Fig. 1).

In Vitro Studies of Melatonin

Five different concentrations (10^{-8} M–10 mM) of melatonin were added to the enzyme activity determination medium which containing bromothymol blue, veronal buffer pH 8.6, H_2O, enzyme solution, melatonin and CO_2-saturated H_2O (total volume 4.2 ml). HCA-I and HCA-II izoenzymes activities with melatonin were assayed by following the CO_2-hydration method.[23] Activity (%) values of HCA-I and HCA-II for five different concentrations of melatonin were drawn by using regression analysis graphs. Carbonic anhydrase activity without melatonin was taken as 100% activity.

In Vivo Studies of Melatonin

Twenty adult male Spraque–Dawley rats (200–250 g) were used for the experiment. All of the animals were fed with standard laboratory chow and water before the experiment. The animal laboratory was windowless with automatic temperature (22 ± 1°C) and lighting controls (14 h light/10 h dark). The animals were divided into two equal groups of ten animals. Animals were pretreated with 10 mg/kg dose of melatonin. Control animals received an equal volume of 0.9% NaCl. All of the animals were housed in different cages.

For control, 0.5 mL of blood sample was taken from tail vein before drug administration. Then, 10 mg/kg

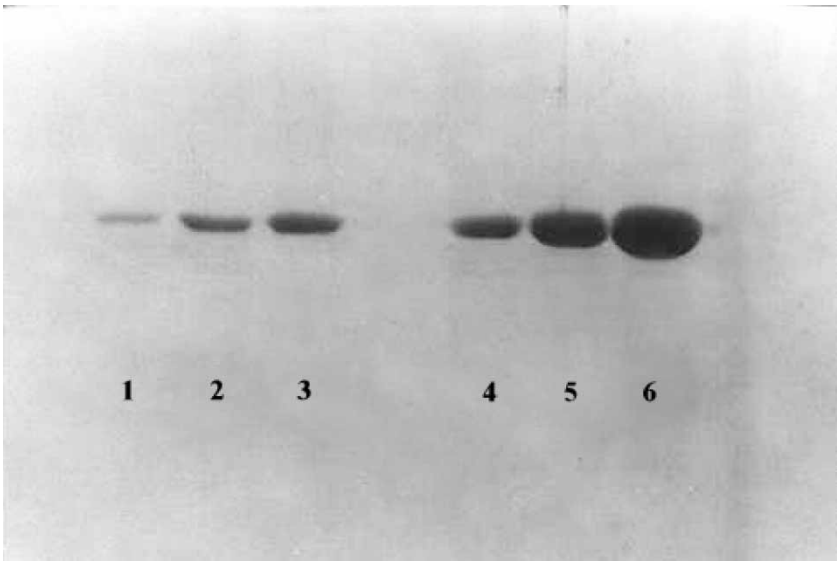

FIGURE 1 SDS-polyacrylamide gel electrophoresis of HCA-I and HCA-II purified by affinity gel. Lane 1,2 and 3 are HCA-I; Lane 4,5 and 6 are HCA-II. (HCA: Human erythrocyte carbonic anhydrase).

melatonin was intraperitoneally administered to the rats; 1,3 and 6 h after drug administration, 0.5 mL blood samples were taken again from tail vein. All of the blood samples were added to test tubes with EDTA. Haemolysate was prepared as described for the *in vitro* studies. CA activity was measured at 37°C using the CO_2-hydration method.[23]

Statistical Analysis

All the *in vivo* results are given as mean ± S.D. of eight parallel measurements. Analysis of variance was performed by t-test. P values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Melatonin originally found in the pineal gland, as well as other organs, attracted much interest after its antioxidant ability was proven both by *in vivo* and *in vitro* studies.[3,4,26–28] Melatonin exists in all body fluids after its release from the pineal. It is often utilized, as a sleep-inducing agent being an important hormone.[29] The pineal hormone has influence on a variety of biological processes including circadian rhythms, neuroendocrine, cardiovascular and immune functions, as well as for thermoregulation.[2,3] Additionally, melatonin functions in protecting cell components such as nuclear DNA, membrane lipids and cytosolic proteins from free radical damage.[18–21]

Many chemicals when administered at relatively low doses affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme.[30] The effects can be dramatic and systemic.[31] Therefore, melatonin has an important role by altering the activities of enzymes used to improve the total antioxidative defence capacity of the organism.[17] Its impact on carbonic anhydrase (CA) activity has not previously been reported. Carbonic anhydrase is a very important enzyme in the body and all the CA isozymes are deeply involved in a great number of secretory activities including fluid movements.[5,32] The physiological function of the CA isozymes is important in facilitating the interconversion of CO_2 and HCO_3^-; also, they play key roles in physiological pH control in most tissue.[8] Therefore, in this study, the investigation of the effects of increasing

TABLE I Purification scheme for HCA-I and HCA-II from human erythrocytes

Steps		Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Haemolysate		975	150	112	16800	146250	8.70	100	1
Sepharose	HCA-I	5870	15	0.80	12	88050	7337.5	60	843
4B-L tyrosine sulfanilamide affinity chromatography	HCA-II	2560	13	0.15	1.95	33280	17067	22.7	1962

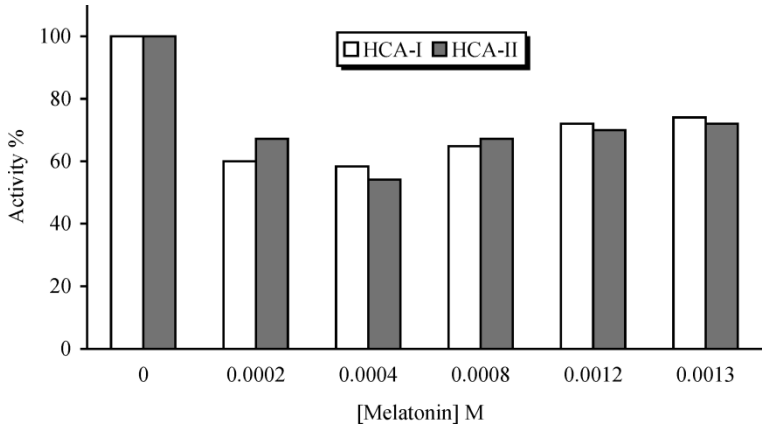

FIGURE 2 Activity%-[Melatonin] graph for HCA-I and HCA-II (HCA: Human erythrocyte carbonic anhydrase).

concentrations of administered melatonin on human erythrocyte HCA-I and HCA-II isozymes was undertaken. In our study, HCA-I and HCA-II isozymes were separately purified from human erythrocytes by Sepharose 4B-L-tyrosine-sulfanylamide affinity column chromatography. The purification process for HCA-I and HCA-II is summarized in Table I. Overall, the purification process showed a recovery of 60% and 22.7% and purification of 843-fold and 1962-fold, respectively. Specific activity was calculated as 7337.5 and 17067 EU/mg proteins for the HCA-I and HCA-II isozymes, respectively (Table I). Figure 1 exhibits the SDS-PAGE made according to Laemmli's procedure to determine the purity of the each isozyme.[25]

For the *in vitro* study, nine different melatonin concentrations were used to determine the effects on human CA-I and CA-II isozymes. As can be seen from Figure 2, it was evident from these *in vitro* studies that the HCA-I and HCA-II were inhibited up to 2×10^{-4} M. However, there is no correlation between inhibition of both CA isoenzymes and the process occuring in a dose-dependent manner. After 4×10^{-4} M concentration of melatonin, the inhibitory effects of melatonin decreased with increased melatonin concentration (Figure 2). For the *in vivo* studies, 20 adult (200–250 g) Sprague–Dawley rats were selected. The CA activity of the control group was found to be 2660 ± 135.8 EU/gHb. The melatonin injection was performed on the control groups intraperitoneally. After the melatonin injection, the enzyme activities of the groups were measured at 1, 3 and 6 h. The corresponding activities were observed as 2500 ± 500.0 ($p > 0.05$), 1875 ± 239.40 ($p < 0.05$) and 2666 ± 235.70 ($p > 0.05$), respectively the greatest inhibition being found at 3 h after injection (Table II).

TABLE II Effect of intraperitoneal melatonin administration (10 mg/kg body weight) on rat carbonic anhydrase activity

Time after administration (h)	CA activity (EU/g Hb)[1]
0	2660 ± 235.8[a]
1	2500 ± 500.0[a,b]
3	1875 ± 239.4[a,b]
6	2666 ± 235.7[a]

[a] Results are given as mean ± S.D. of eight parallel measurements. [b] These values were regarded as significant ($p < 0.05$). Analysis of variance was performed by t-test.

In conclusion, HCA-I and HCA-II was purified from human erythrocytes. CA is widely distributed in most tissues and has a very important role in some diseases such as glaucoma, where CA inhibitors are generally used for treatment since they reduce intraocular pressure. Our study revealed that there was a good correlation between the *in vivo* and *in vitro* inhibitory effects of melatonin on HCA-I and HCA-II activities. Melatonin has a function in protecting cell components such as nuclear DNA, membrane lipids, and cytosolic proteins from free radical damage.

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