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Effects of low molecular weight plasma inhibitors of rainbow trout (*Oncorhynchus mykiss*) on human erythrocyte carbonic anhydrase-II isozyme activity *in vitro* and rat erythrocytes *in vivo*

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Abstract

The effects of low molecular weight plasma inhibitors from rainbow trout (*Oncorhynchus mykiss*) (RT) were investigated on the carbonic anhydrase enzyme (CA) activities in *in vitro* human and in *in vivo* Sprague–Dawley rat erythrocytes. The RT blood was used as extracellular fluid (plasma) source and plasma inhibitors were obtained by dialysis of the plasma. For the *in vitro* study, human carbonic anhydrase-II (HCA-II) isozyme was obtained by Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography with an overall purification of about 646-fold. The enzyme (specific activity of 7750 EU/mg protein) was obtained with a yield of 71.1% and SDS-PAGE showed a single band. From *in vitro* studies, the I_{50} value for RT plasma inhibitors obtained was 0.37 mg/ml. From *in vivo* studies on rat erythrocytes, CA activity was significantly inhibited by the inhibitors from the extracellular fluid of RT for up to 3 h ($p < 0.05$) following intraperitoneal administration.

Keywords: *Carbonic anhydrase, rainbow trout, inhibition, plasma inhibitors*

Abbreviations: *CA, carbonic anhydrase; RT, rainbow trout*

Introduction

Carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) is a ubiquitous zinc-containing enzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+. This enzyme, involved in a wide range of physiological and biochemical processes, is present in most tissues including erythrocytes. The enzyme has been well characterized as a pH regulatory enzyme in many different tissues [1–5].

Up to the present, fourteen different CA isozymes have been described in higher vertebrates [6,7]. Among CA isozymes are cytosolic (such as CA I, CAII, CA III, CA VII), membrane-bound (CA IV, CA IX, CA XII and CA XIV), mitochondrial (CA V) and secretory forms (CA VI) as well as several acatalytic forms (CA VIII, CA X and CA XI) [8–10].

CA inhibitors, especially aromatic and heterocyclic sulfonamides, have been used clinically for a long time in the treatment of some diseases, for example, glaucoma, acid–base disequilibria, gastric and duodenal ulcers. In addition, the inhibitors have been applied as antitumor agents [11–13].

The CA enzyme activity in the plasma of lower vertebrates is minimal, the cause of this situation being the presence of naturally occurring inhibitors for this enzyme [1]. Henry et al. [14] have measured the presence of inhibitors in plasma and subcellular fractions of gill tissue in the subclass of fishes as elasmobranches (cartilaginous fishes comprising the sharks and rays) and teleost (bony fishes). Additionally, they reported that plasma CA inhibitors were highly species-specific in salmonids (pertaining to the family Salmonidae, including the salmons,

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trouts, chars, and whitefishes) and selective for scavenging the zinc metal ion associated with the active site of CA released during hemolysis. However, we have not encountered any literature concerning the number of RT plasma CA inhibitors, their structure and effects on human CA enzyme activity.

In conclusion, many CA inhibitors have been and are still used clinically in the treatment of certain diseases, but naturally-occurring inhibitors of these enzymes have been rarely investigated up to now, and they may find clinical applications if studied in more detail. We investigate here the effects of RT low molecular weight plasma inhibitors on carbonic anhydrase enzyme activities in *in vitro* human and *in vivo* rat erythrocytes.

Materials and methods

Materials

Sepharose 4B activated by CNBr, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). 4-Aminobenzene sulfonamide and L-tyrosine were from E. Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of the hemolysate and hemoglobin estimation

Fresh human blood was obtained from the Atatürk University Hospital Blood Center. The haemolysate was prepared according to a previous study [15]. Hemoglobin (Hb) concentration in the hemolysate was determined by the cyanmethemoglobin method [16–18]. All studies were performed at 4°C.

Purification of HCA-II from human erythrocytes by Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography

The pH of the hemolysate solution obtained previously was adjusted to pH 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 carbonic anhydrase-II isozyme 19]. HCA-II isozyme was eluted with 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), (flow rate: 20 ml h^{-1}, fraction volume: 4 ml) [4,20]. During the HCA-II isozyme purification procedure, the absorbency at 280 nm was used to monitor protein elution by affinity chromatography. CO_2-hydratase activities in the eluates were determined and the active fractions were collected [3,21].

Determination of HCA-II activity

Enzyme activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson [21] modified by Rickli *et al.* [22] CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_o - t_c)/t_c$ Where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Protein determination

The absorbance at 280 nm was used to monitor the protein in the column effluents. Quantitative protein levels were determined spectrophotometrically (595 nm) according to the Bradford method, using bovine serum albumin as standard [23].

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity was carried out using Laemmli's procedure [24] with 4% and 10% acrylamide concentrations for running and stacking gel, respectively. The gel solution was supplemented with 10% SDS.

Fish husbandry and maintenance

The rainbow trout obtained from the Fisheries Department of the Agricultural Faculty at Atatürk University in Erzurum were one year-old (mean wt 200 ± 20 g). Aerated dechlorinated tap water with a constant water flow of 1.5 l/min, 9 ± 1°C average water temperature, 9 ppm dissolved oxygen, 7.8 pH and 102 mg as $CaCO_3$ total hardness was used in the experiment [3]. A commercial pellet diet with 49.4% protein, 18.2% fat, 94.3% dry matter, and 9.8% ash was used to feed the fish during the study.

Obtaining of RT plasma inhibitors and lyophilization

For plasma inhibitors, firstly, the animals were anesthetized (MS-222 was used), blood was collected via heart puncture with a syringe flushed with EDTA and then placed into EDTA-containing tubes [25]. These samples were centrifuged (15 min, 3000 × g) (MSE, MISTRAL 2000) and plasma was taken and dialyzed against deionized water. In this manner the RT plasma inhibitors passed into the dialysis solution. Then, the dialyzate was removed. The dialysis solution was used for lyophilization. For this purpose, the dialysis solution was filtered over Whatman No. 1 paper, then the filtrate was frozen and lyophilized

in a lyophilizator at 5 mmHg pressure at −50°C (Labconco, 104 Freezone 1L) [26]. The lyophilized solution was placed in a plastic bottle and stored at −20°C until used.

In vitro *inhibition studies*

In order to determine the effects of RT plasma inhibitors on HCA-II isozyme, six different concentrations [0.165, 0.33, 0.495, 0.66, 0.908 and 0.99 mg/ml] were added to separate tubes containing 50 μl of purified enzyme and the enzyme activity was measured. Carbonic anhydrase activity without the plasma inhibitors solution was accepted as a 100% activity. The inhibitor concentration causing up to 50% inhibition was graphically determined.

The mathematical relationship between RT plasma inhibitors concentrations and CA activities was determined using conventional polynomial regression software (Microsoft Office 97, Excel).

In vivo *inhibition studies*

Ten adult Sprague–Dawley rats (wt 200 ± 20 g) were selected for intraperitoneal (i.p.) administration of lyophilized RT plasma inhibitors (50 mg kg^{-1}) [4]. Blood samples (0.5 ml) were taken from each rat prior to plasma inhibitors solution administration as well as at 1, 3 and 5 h thereafter. They were collected into test tubes containing EDTA (2 mM) and centrifuged at 2500 × g for 15 min at 4°C. The erythrocyte pellet was washed three times with cold 0.9% NaCl and the supernatant was discarded. One volume of erythrocyte pellet was suspended in five volumes of ice-water to give an erythrocyte hemolysate. CA activity was determined using the CO_2-hydration method [23].

Statistical analysis

The obtained data were subjected to statistical analysis applying a one way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine significant difference among means at the $\alpha = 0.05$ level. Acceptable significance was recorded when P values were < 0.05.

Results

The procedure of Arslan *et al.* [19] was followed to purify the enzyme HCA-II from human erythrocytes and the process is summarized in Table I. In the first step the prepared hemolysate was adjusted to pH 8.7 with solid Tris and than Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography was followed. The elution profile of Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography is shown in Figure 1. HCA-II was purified with a specific activity of 7750 EU/mg protein and approximately 646-fold in a 71.1% yield (Table I). The purity of the enzyme was confirmed by SDS-PAGE (Figure 2). Figure 3 shows the *in vitro* effects of the RT plasma inhibitors on the enzyme activity. I_{50} values were estimated at 0.37 mg/ml. The results of *in vivo* effects of RT plasma inhibitors are presented in Table II. In the RT plasma inhibitors solution treated group, the control enzyme activity was 28387.25 ± 749.084 EU$(gHb)^{-1}$, while the respective values determined 1, 3 and 5 h after RT plasma inhibitors administration were 23486.75 ± 1974.570 ($p < 0.05$), 19066.60 ± 2227.78 ($p < 0.05$), and 26840.40 ± 1425.08 EU$(gHb)^{-1}$ ($p > 0.05$), respectively. The greatest inhibition was found 3 h after injection (Table II).

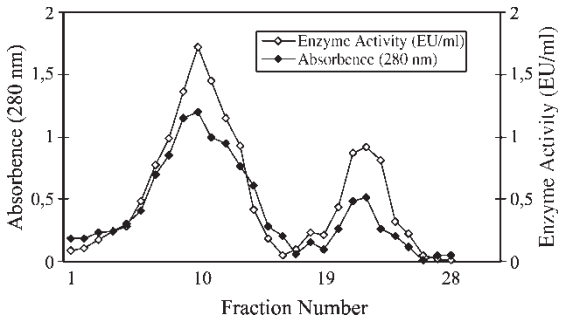

Figure 1. Elution graph of HCA-I (first fraction) and HCA-II (second fraction) isozymes from human erythrocytes with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), respectively (flow rate: 20 ml h^{-1}, fraction volume: 4 ml).

Discussion

It is generally recognized that CA (EC 4.2.1.1) controls the bulk of CO_2 exchange between blood and tissues as well as the regulation of H^+ and other ion movements between cells and extracellular fluids. Moreover, all CA isozymes are also involved in a large number of secretory activities including fluid movements [27]. CA is a physiologically very important enzyme since it is present not only in erythrocytes but

Table I. Purification Scheme for HCA-II from Human Erythrocytes.

Purification Step	Activity (EU/ml)	Total Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg)	Yield (%)	Purification Factor
Hemolysate	1453	150	121	18150	217950	12	100	1
Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography	6200	25	0.8	20	155000	7750	71.1	646

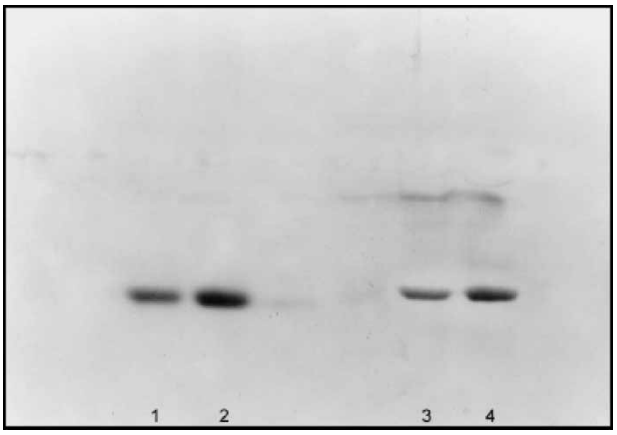

Figure 2. SDS-Polyacrylamide gel electrophoresis of HCA-I and HCA-II purified by Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography. (Lane 1 and 2 are HCA-I; Lane 3 and 4 are HCA-II).

also in many tissues, being also involved in the metabolism [28]. Therefore, CA has been a long established target for drug design with several sulfonamides of the type RSO_2NH_2 being used clinically since 1956, when acetazolamide, the first of these was approved as a diuretic. [1,27] Sulfonamides are now widely used drugs for the treatment or prevention of a variety of diseases, such as glaucoma, gastroduodenal, ulcers, acid-base disequilibria and diverse neurological/neuromuscular disorders or tumors. [6,11,13,27]

In previous work we showed the effects of sodium ampicillin, magnesium sulfate, sodium dipyrone on human erythrocyte CA activity *in vitro* and rat erythrocyte CA activity *in vivo* [4]. Additionally, we have reported that the catalytic activity of

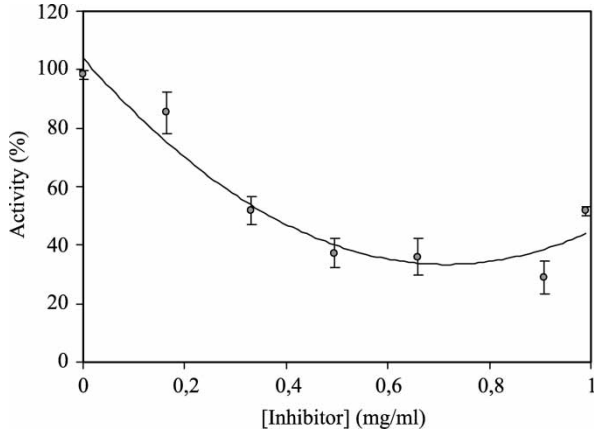

Figure 3. Effect of plasma inhibitors concentrations (0.16, 0.33, 0.50, 0.66, 0.91 and 0.99 mg/ml) on human erythrocyte CA-II activity. Experimental analysis were performed at 4°C. CA enzyme activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson.[21] The enzyme unit (EU) was calculated by using the equation $(t_o - t_c/t_c)$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Table II. Statistical values obtained from *in vivo* studies on rat erythrocyte CA for the RT plasma inhibitors.

Compound	Time	X ± SD EU(g Hb)$^{-1}$
RT plasma inhibitors	Control	28387.25 ± 749.084[a]
	1 h	23486.75 ± 1974.570[b]
	3 h	19066.60 ± 2227.78[c]
	5 h	26840.40 ± 1425.08[a]

Means having different superscripts in a column differ significantly ($p < 0.05$).

the enzyme is inhibited by gentamicin sulfate, an important therapeutic antibiotic [29]. Although, the inhibitory effects of many chemicals and therapeutic drugs on the enzyme have been studied in most tissues and red blood cells, no study has yet been done on the effects of RT plasma inhibitors on human CA-II isozyme. Human erythrocyte CA-II purified enzyme (Table I and Figure 1) was used for this purpose. The I_{50} value obtained for the plasma inhibitor isolated from RT was 0.37 mg/ml on the HCA-II isozyme. Inhibitory effects of the substances present in RT plasma against HCA-II may be due to their binding to the active site of the enzyme or to elsewhere on the protein, possibly interacting with the access of substrates within the active site cavity.

During the *in vivo* studies performed on the rat, the highest inhibition was found 3 h after injection (Table II). This result indicates that the inhibitor molecules in the RT plasma entered into the erythrocytes. If RT plasma inhibitors at a dosage of 50 mg/kg were administered i.p. in humans (wt 60 kg, c. 5 L blood volume) a plasma inhibitors concentration of approximately 0.6 mg/ml would be achieved. This concentration is within the range (0.4–0.8 mg/ml) which is inhibitory against *in vitro* CA-II activity (Figure 2).

In conclusion, the effects of RT low molecular weight plasma inhibitors on carbonic anhydrase enzyme activities in *in vitro* human and *in vivo* rat erythrocytes showed that RT plasma inhibitors had an inhibitory effect *in vitro* on HCA-II and *in vivo* on rat CA. Future work is needed to purify these inhibitors from RT plasma and to determine their precise chemical nature.

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