

EXPERIMENTAL STUDY

The effects of some tumor markers on human erythrocyte (HCA-I and HCA-II), bovine erythrocyte (BCA) and bovine lung (CA-IV) carbonic anhydrase enzyme activities *in vitro*

Demir N¹, Nadaroglu H², Gungor AA³, Demir Y^{1,4}

Faculty of Sciences, Chemistry Department, Mugla University, 48000, Mugla, Turkey. demirn@yahoo.com

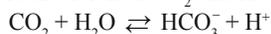
Abstract: *Objective:* The influence of prostatic acid phosphatase (PAP) and human chorionic gonadotropin (HCG), tumor markers have been investigated on human erythrocyte carbonic anhydrase (HCA-I and HCA-II) and bovine erythrocyte (BCA) and bovine lung carbonic anhydrase (CA-IV) *in vitro*.

Background: Tumor markers are substances that can often be detected in higher-than-normal amounts in the blood, urine, or body tissues of some patients with certain types of cancer. Tumor markers are produced either by the tumor itself or by the body in response to the presence of cancer or certain benign (noncancerous) conditions. In addition to their role in cancer diagnosis, some tumor marker levels are measured before treatment to help doctors plan appropriate therapy.

Results and conclusion: All of the tumor markers were determined to have inhibition effect, on human CA-I, CA-II, bovine erythrocyte CA (BCA) and bovine lung CA-IV isoenzymes. The effect of each tumor marker on CA was investigated by Wilbur-Andersen method modified by Rickly et al Inhibition effects of two different tumor markers on human CA-I, CA-II, bovine erythrocyte CA (BCA) and bovine lung CA-IV isoenzymes were determined by using the CO₂-Hydratase method by plotting activity % vs (tumor markers). I₅₀ values of tumor markers exhibiting inhibition effects were found by means of these graphs (Tab.1, Fig. 2, Ref. 20). Text in PDF www.elis.sk. Key words: tumor markers, carbonic anhydrase, HCA-I, HCA-II, BCA, CA-IV, Zn²⁺.

Introduction

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) isozymes are a family of zinc metalloenzymes which catalyze the interconversion of CO₂ and HCO₃⁻ (1).



The enzyme, discovered about 70 years ago (2, 3), is abundantly present in mammalian red blood cells and to a lesser extent in different types of tissues and secretory organs (5–6). In addition, carbonic anhydrase from plant, yeast and bacteria have been reported and partially characterized (5–11). The important roles of the enzyme in various cell types have been extensively reviewed (5, 6). At present, approximately 14 isozymes (I–XIV) are reported and found to be distributed throughout the living organisms (5, 6, 11).

In man, CA-IV has been identified in the microvilli and basal infoldings of renal tubular cells, and this isoform has recently

been purified and characterized (12). An apparently homologous enzyme (13) has also been purified from human lung. It is proposed that the CA-IV isoenzyme is primarily responsible for the re-absorption of HCO₃⁻ in the proximal tubule and is involved in the formation of lung liquid during fetal life.

The carbonic anhydrase enzyme taken from cow lung has already been partially purified (12). The process of purification carried out at our laboratory produced some new and significant results. The present study made it possible to find new data about CA-IV enzyme which is of vital importance for respiration which would otherwise be left incomplete. A new definition for this enzyme is presented as a result of our experiment.

The critical enzyme can be thought to be affected by tumor markers, which are widely consumed. This *in vitro* study was planned in order to investigate the effect of tumor markers with or without alcohol on the enzyme, which is important in respect of human health.

Materials and methods

Purification of carbonic anhydrase from human erythrocytes

Erythrocytes were purified from human blood obtained from Blood Center of Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 20 min and the plasma and buffy coat were removed. After the packed red cells had been washed with NaCl (0.9 %) three times, the erythrocytes

¹Faculty of Sciences, Chemistry Department, Mugla University, Mugla, Turkey, ²Department of Food Technology, Erzurum Vocational Training School, Atatürk University, Erzurum, Turkey, ³Department of Chemical Technology, Erzurum Vocational Training School, Atatürk University, Erzurum, Turkey, and ⁴Department of Chemistry, Faculty of Science, University of Mehmet Akif Ersoy, Burdur, Turkey

Address for correspondence: N. Demir, MD, Mugla University, Faculty of Sciences, Chemistry Department, 48000, Mugla, Turkey. Phone: +90.442.2314439, Fax: +90.442.2360948

were hemolyzed with cold water. The ghost and intact cells were removed by centrifugation at 4 °C, 20,000 rpm for 30 min. The pH of hemolysate was brought to 8.5 with solid tris. The hemolysate was applied to the affinity column having a structure of Sepharose 4B-L-tyrosine-sulfonyamide and equilibrated with 25 mM Tris-HCl / 0.1 M Na₂SO₄ (pH 8.5). The affinity gel was washed with a solution of 25 mM Tris-HCl / 22 mM Na₂SO₄ (pH 8.5). HCA-I and HCA-II isoenzymes were eluted with a solution of 1 M NaCl / 25 mM Na₂HPO₄ (pH 6.3) and 0.1 M NaCH₃COO / 0.5 M NaClO₄ (pH 5.6) (14). The elutions were plotted by protein determination at 280 nm and CO₂-hydratase activity and the purification was controlled with SDS-PAGE (15).

Preparation of carbonic anhydrase from bovine erythrocytes

Bovine blood was anticoagulated with 1.5 mL of ACD solution (22 g of sodium citrate dihydrate, 8 g of citric acid and 24 g of dextrose per liter) per 100 mL blood, and red blood cells were obtained and washed with saline. The cells were lysed with distilled water. The ghosts were removed by centrifugation. The hemolysate was applied to the affinity column, having a structure of Sepharose 4B-L-tyrosine-sulfonyamide and equilibrated with 25 mM Tris-HCl / 0.1 M Na₂SO₄ (pH 8.5). The affinity gel was washed with a solution of 25 mM Tris-HCl / 22 mM Na₂SO₄ (pH 8.5). BCA was eluted with a solution of 0.1 M NaCH₃COO / 0.5 M NaClO₄ (pH 5.6) (14). The elutions were plotted by doing protein determination at 280 nm and CO₂-hydratase activity and the purification was controlled with SDS-PAGE (15).

Purification of carbonic anhydrase from bovine lung

Samples about 250 g in weight from cow lung were taken during slaughtering at the Erzurum Municipal Slaughterhouse and rinsed into a solution of 0.9 % NaCl. Firstly, a piece of lung was cut into smaller pieces, washed with physiological serum and then chopped in a blender. Approximately 60 liters of serum was used in order to eliminate erythrocytes from the medium. Washing was continued until achieving precipitation that was clear enough.

The sample of lung, entirely free of erythrocytes, was taken into 500 mL buffer of 0.05 M Tris-SO₄ (pH 7.4) containing 1 % Triton X-100 (2 mL per gram), dismembered by an ultrasonic dismembrator for 4 hours and then centrifuged for 30 minutes at 3000xg. The precipitation was removed by filtering through fiberglass. The supernatant was treated with distilled water for 2 days and with 0.05 M Tris- SO₄ (pH 7.4) buffer for dialysis. Then the homogenate was extracted by approximately 500 mL CCl₄ to eliminate the lipids from the medium and organic medium was removed. The pH of homogenate was brought to 8.7 by using solid Tris until it was prepared to be applied to column. The same af-

finity gel was used for CA-IV. CA-IV was eluted with a solution of 0.1 M NaCH₃COO / 0.5 M NaClO₄ (pH 5.6) (13, 14). Protein concentration and activity were determined (16). Purification was controlled with SDS-PAGE (15).

Determination of carbonic anhydrase activity and effect of tumor markers on isoenzymes

Carbonic anhydrase activity and effect of tumor markers were assayed by hydration of CO₂ measured by the method of Rickli et al (17) and Wilbur-Anderson with bromothymol blue as indicator. CO₂-Hydratase activity as enzyme unit (EU) was calculated by the equation ($t_0 - t_c / t_c$) where t_0 and t_c are the times for pH change in non-enzymatic (buffer) and enzymatic reaction, respectively.

Determination of I₅₀ values

The values of I₅₀ (inhibitor concentration reduced the enzyme activity by 50 %) have been determined graphically using five different inhibitor concentrations. For hormones showing inhibition effect, the values of I₅₀ with diagram of activity percent-(I) were calculated.

Results

Many chemicals at a relatively low dosage affect the metabolism of biota by altering normal enzyme activity, particularly inhibiting specific enzymes (18). The effects can be dramatic and systemic (19). Although prostatic acid phosphatase (PAP) and human chorionic gonadotropin (HCG) are used, reduction in marker concentration indicates effective therapy, and increase in concentration marks tumor progression, there is no carbonic anhydrase activity for these tumor markers used in our study. Indeed, CA isoenzymes are important enzymes for body metabolism because they regulate pH in most tissue. Therefore, in the present study, investigation of effects of certain tumor markers on human erythrocyte carbonic anhydrase I and II, bovine erythrocyte carbonic anhydrase (BCA) and bovine lung carbonic anhydrase (CA-IV) isoenzymes was proposed. In order to conduct this study, HCA-I, HCA-II separately from human erythrocytes, BCA from bovine erythrocytes and CA-IV from bovine lung were purified by Sepharose 4B-L-tyrosine-sulfonyamide affinity chromatography. Prostatic acid phosphatase (PAP) and human chorionic gonadotropin (HCG) as tumor markers were chosen for the investigation of inhibition or activation effects.

For the tumor markers exhibiting the effect, the inhibitor concentrations causing up to 50 % inhibition (I₅₀ values) were determined from the regression analysis graph. I₅₀ values obtained for of HCA-I, HCA-II, CA-IV and BCA are shown in Table 1.

To show inhibition effects, values of HCA-I, HCA-II, BCA and CA-IV for seven different concentrations of each tumor markers were determined (Figs 1 and 2).

Discussion

Prostatic acid phosphatase (PAP) is normally present only in small amounts in the blood, but may be found at higher levels in

Tab. 1. The results of inhibition studies for HCG and PAP compounds on HCA-I, HCA-II, CA-IV and BCA.

Compound	HCA-I I ₅₀ (nM)	HCA-II I ₅₀ (nM)	CA-IV I ₅₀ (nM)	BCA I ₅₀ (nM)
HCG	6.78x10 ⁻²	6.79 x10 ⁻²	3.78 x10 ⁻²	2.59 x10 ⁻²
PAP	6.19x10 ⁻²	6.08 x10 ⁻²	2.66x10 ⁻²	1.55x10 ⁻²

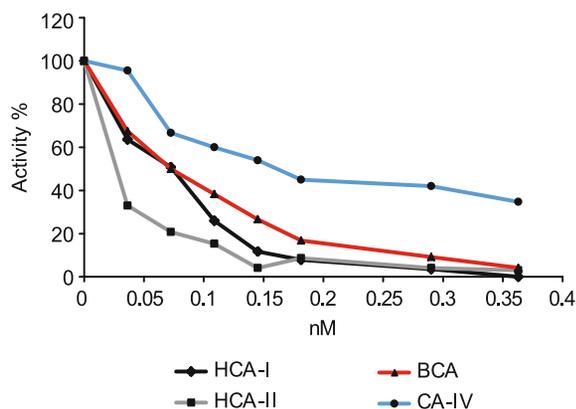


Fig. 1. The effect of prostatic acid phosphatase (PAP) on activity of carbonic anhydrase isoenzymes (HCA-I, HCA-II, BCA and CA-IV).

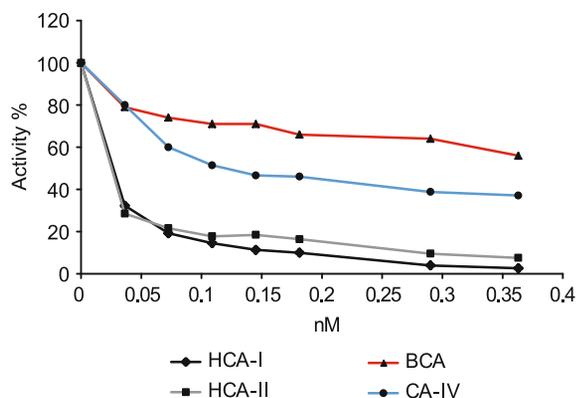


Fig. 2. The effect of human chorionic gonadotropin (HCG) on activity of carbonic anhydrase isoenzymes (HCA-I, HCA-II, BCA and CA-IV).

some patients with prostate cancer, especially if the cancer has spread beyond the prostate. However, blood levels may also be elevated in patients who have certain benign prostate conditions or early stage cancer. Although PAP was originally found to be produced by the prostate, elevated PAP levels have since been associated with testicular cancer, leukemia, and non-Hodgkin's lymphoma, as well as noncancerous conditions such as Gaucher's disease, Paget's disease, osteoporosis, cirrhosis of the liver, pulmonary embolism, and hyperparathyroidism.

Human chorionic gonadotropin (HCG) is normally produced by the placenta during pregnancy. In fact, HCG is sometimes used as a pregnancy test because it increases early within the first trimester. It is also used to screen for choriocarcinoma (a rare cancer of the uterus) in women who are at high risk for the disease, and to monitor the treatment of trophoblastic disease (a rare cancer that develops from an abnormally fertilized egg). Elevated HCG levels may also indicate the presence of cancers of the testis, ovary, liver, stomach, pancreas, and lung. Pregnancy and marijuana use can also cause elevated HCG levels.

It is generally recognized that CA controls the bulk of carbon dioxide exchange between blood and tissues as well as regulation

of proton and other ion movements between cells and extracellular fluids. All of the CA isoenzymes are also deeply involved in a great number of secretory activities including fluid movements (20).

Conclusion

Since CA is a very important enzyme for the body, the inhibition effects of these tumor markers should be considered not only for HCA-I, HCA-II, BCA and CA-IV isoenzymes but also for all CA isoenzymes.

Tumor markers showing inhibitory effects on HCA-I, HCA-II, BCA and CA-IV isoenzymes increase their effect bound to active center of enzymes or other part of enzymes.

The HCG compounds have higher I_{50} values than PAP on HCA-I, HCA-II, CA-IV and BCA. By comparing I_{50} values of the compounds, it has been found that compound HCG is a more potent inhibitor than PAP on HCA-I, HCA-II, CA-IV and BCA.

References

- Hewett-Emmett D, Tashian RE. Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. *Mol Phylogenet Evol* 1996; 5: 50–77.
- Meldrum NU, Roughton FJW. Carbonic anhydrase: Its preparation and properties. *J Physiol* 1933; 80: 113–142.
- Stadie WC, O'Brien H. The catalysis of the hydration of carbon dioxide and dehydration of carbonic acid by the enzyme isolated from red blood cells. *J Biochem* 1933; 103: 521–529.
- Lindskog S. Carbonic anhydrase. *Adv Inorg Biochem* 1982; 4: 115–170.
- Dodgson SJ. The carbonic anhydrases: overview of their importance in cellular physiology and in molecular genetics. In: Dodgson SJ, Tashian RE, Gros G, Carter ND (Eds). *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*. New York: Plenum Press, 1991; 3–14.
- Sigler K, Hofer M. Mechanism of acid extrusion in yeast. *Biochim Biophys Acta* 1991; 1071: 375–391.
- Veitch FP, Blankenship LC. Carbonic anhydrase in bacteria. *Nature* 1963; 197: 76–77.
- Shoaf WT, Jones ME. Carbonic anhydrase of microorganisms I: an enzyme from baker's yeast which catalyzes the formation of carbamate from ammonium bicarbonate solutions. *Arch Biochem Biophys* 1970; 139: 130–142.
- Mitsuhashi S, Miyachi S. Amino acid sequence homology between N- and C-terminal halves of a carbonic anhydrase in *Porphyridium purpureum*, as deduced from the cloned cDNA. *J Biol Chem* 1996; 271: 28703–28709.
- Mulligan ME, Coleman JR. Identification and characterization of a gene encoding a vertebrate-type carbonic anhydrase in cyanobacteria. *J Bacteriol* 1997; 179: 769–774.
- Fujikawa AK, Nishimori I, Taguchi T, Onishi S. Human carbonic anhydrase XIV (CA14): cDNA cloning, mRNA expression, and mapping to chromosome 1. *Genomics* 1999; 61: 74–81.
- Carter ND, Fryer A, Grand AG, Hume R, Strange RG, Wistrand PJ. Membrane specific carbonic anhydrase (CA-IV) expression in human tissues. *Biochem Biophys Acta* 1990; 1026: 113–116.

- 13. Zhu XL, Sly WS.** Carbonic anhydrase IV from human lung. Purification, characterization and comparison with membrane carbonic anhydrase from human kidney. *J Biol Chem* 1990; 265: 8795–8801.
- 14. Arslan O, Nalbantoglu B, Demir N, Ozdemir H, Kufrevioglu OI.** A new method for the purification of carbonic anhydrase isoenzymes by affinity chromatography. *Turk J Med Sci* 1996; 26: 163–166.
- 15. Laemmli UK.** Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* 1970; 227: 680–685.
- 16. Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annal Biochem* 1976; 72: 248–254.
- 17. Rickli EE, Ghazantar SAS, Gibbons BH, Edsall JT.** Carbonic anhydrase from human erythrocytes. *J Biol Chem* 1964; 239: 1065–1078.
- 18. Hoehster RM, Kates M, Questel JH.** Metabolic inhibitors. Vols 3 and 4 New York: Academic press, 1972; 71–89, 1973; 66–82.
- 19. Christensen GM, Olson D, Riedel B.** Chemical effects on the activity of eight enzymes: A review and discussion relevant to environmental monitoring. *Environ Res* 1982; 29: 247–255.
- 20. Botre F, Botre C. 1991:** Physiologic implications of carbonic anhydrase facilitated carbon dioxide diffusion coupling to other biometabolic processes. In: Botre FG, Gros, Storey BT (Ed). *Carbonic Anhydrase: From Biochemistry and Genetics to Physiology and Clinical Medicine; International Workshop On Carbonic Anhydrase, Spoleto, Italy, March 4–7.* Xvi 467 p.

Received April 6, 2014.
Accepted January 20, 2015.