FULL PAPER *Physiology*

Measurement of Erythrocyte Carbonic Anhydrase Isozymes (CA-I and CA-II) in Racehorses and Riding Horses

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ABSTRACT. Equine carbonic anhydrase isozymes (CA-I and CA-II) were purified from erythrocytes by several column chromatography. Polyclonal anti-CA-I and anti-CA-II sera were produced in rabbits. Sensitive competitive enzyme-linked immunosorbent assays (ELISA) were established to determine the developmental changes in CA-I and CA-II levels in equine erythrocytes. Concentrations of CA-I and CA-II in erythrocytes from 150 clinically normal thoroughbreds (123 racehorses and 27 riding horses) were determined by ELISA. Mean (± SD) concentrations of CA-I and CA-II in racehorses were 1.70 ± 0.48 and 0.94 ± 0.13 mg/g hemoglobin (Hb), respectively. Mean concentrations of CA-I and CA-II in riding horses were 2.34 ± 0.52 and 0.76 ± 0.08 mg/g Hb, respectively. When the CA levels in racehorses and riding horses were compared, the CA-I level in riding horses was higher than that in racehorses (p=0.01). The CA-II level in racehorses was higher than that in riding horses (p=0.02). These data suggest that the levels of CA isozymes in erythrocytes of racehorses were influenced by chronic physical stress. The CA-I concentration in erythrocytes of 2-month-old horses was approximately 0.25 mg/g Hb. The CA-I level noticeably increased during the first year of life and approached normal adult levels by 2 years. The CA-II level decreased slightly with age, indicating different regulation of CA-I and CA-II expression during development.
KEY WORDS: carbonic anhydrase, equine, ELISA, erythrocyte.

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Carbonic anhydrase (CA; EC 4.2.1.1) catalyzes the hydration of CO_2 and the dehydration of H_2CO_3. Carbonic anhydrase is widely distributed in mammalian tissue and plays important roles in gas transport and acid/base regulation [6]. Eleven CA isozymes (cytosolic CA-I, CA-II, CA-III and CA-IV, mitochondrial CA-V_A and V_A, secretory CA-VI and membrane-associated CA-IV, IX, XII and XIV) are known as active types, whereas three CA-related proteins (CA-VIII, X and XI) are inactive types [7,11]. Each isozyme has a unique molecular structure. Erythrocytes contain mainly CA-I and CA-II isozymes, which are immunologically differentiated [8]. Although CA-II has a 30-fold higher enzymatic activity than CA-I, CA-II accounts for only 11% of total CA activity in human erythrocytes [8].

Furth [10] and Deutsch *et al.* [4] reported that equine erythrocytes contain CA-I and CA-II. We previously reported the immunohistochemical localization of CA-I, CA-II and CA-III in equine salivary glands [2]. We have also immunohistochemically localized CA isozymes to the equine digestive tract [15], but no previous studies have measured CA-I and CA-II levels in equine erythrocytes.

In the present study, we used enzyme-linked immunosorbent assays (ELISA) to determine the developmental changes in CA-I and CA-II levels in equine erythrocytes. ELISA may enable the study of changes in isoenzymes associated with hereditary or metabolic disorders by using only a small sample of blood or other body fluids.

MATERIALS AND METHODS

Purification of CA isozymes: Equine CA-I and CA-II were purified by the method of Deutsch *et al.* [4,5], with minor modifications. As we previously described the purification process in detail [12], briefly blood (500 m*l*) from a clinically normal female thoroughbred (8 years old) was collected in a bottle containing 3.8% citric acid. Erythrocytes were washed by centrifugation with 0.15 M NaCl. The packed erythrocytes were hemolyzed with distilled water. Hemoglobin was extracted by the chloroform/ethanol denaturation method (Tuchihasi extract). The precipitated hemoglobin was removed by centrifugation at 4°C and the supernatant solution was dialyzed against 0.01 M Tris-HCl (pH 8.0) at 4°C. The dialyzed material was applied to a column of inhibitor affinity chromatographic matrix (CM Sephadex coupled to p-aminomethyl benzene sulfonamide) [14] at 4°C. After extensively washing the affinity column with 0.001 M Tris-HCl (pH 8.0), CA isozyme was eluted with 0.001 M Tris-HCl (pH 8.0) containing 0.6 M NaN_3. The fractions that contained CA activity were collected and pooled. The samples were further purified with gel filtration column chromatography and column electrofocusing [18].

The molecular weight of both equine CA-I and CA-II was estimated to be 29,000. The isoelectric point for CA-I and CA-II were 5.7 and 9.3, respectively.

Enzymatic activity was measured by the method of Wilbur and Anderson [20]. Assays were performed at 4°C, and specific activity (U) was determined according to the formula:

$$U = 10X\ (Tb/Te—1)/mg\ of\ protein$$

where Tb is the time required for the uncatalyzed reaction

(ie, pH change from 8.5 to 6.5), and Te is the time required for the enzyme-catalyzed reaction. The specific activities of equine CA-I and CA-II were 3,400 units/mg and 36,000 units/mg, respectively.

Antiserum: Antibodies to purified equine CA isozymes were produced in rabbits. Ten Japanese white rabbits (Jla:JW) were purchased from Japan Laboratory Animals, Inc (Tokyo, Japan). Each rabbit was initially injected with 1 mg of purified CA isozyme emulsified with an equal volume of Freund's complete adjuvant, followed by booster injections of an equivalent amount of CA isozymes once a week for 5 successive weeks. The rabbits were bled through the auricular vein 10 days after the last injection. Antiserum specificity was examined by the double immunodiffusion method.

Antibody was purified from rabbit antiserum on a HiTrap NHS-activated column (Pharmacia Biotech, Uppsala, Sweden) coupled with purified equine CA-I or CA-II. Antibody eluted from the column with 8 M urea was dialyzed against 0.01 M Tris-HCl (pH 8.0).

Biotinylation of carbonic anhydrase: To biotinylate carbonic anhydrase, 2.27 mg of biotin (sulfosuccinimidyl N-[N' (D-biotinyl)-6-aminohexanoyl]-6'-aminohexanoate: Dojindo Laboratories, Kumamoto,) in 0.04 m*l* of 0.01 M HEPES buffer (pH 8.5) was added to a solution containing 5 mg/m*l* of purified CA isozymes. The mixture was incubated at 25°C for 4 hr and then extensively dialyzed against phosphate buffered saline (PBS) solution (pH 7.5).

Erythrocyte samples: Blood samples were collected from 150 clinically normal mature thoroughbreds: 123 racehorses (87 males and 36 females) and 27 riding horses (6 males, 5 females and 16 geldings). The racehorses ranged in age from 2 to 6 years. The riding horses ranged in age from 4 to 23 years and were retired from racing. The riding horses did less intense daily exercises than the racehorses. Blood samples were also collected from 12 immature thoroughbreds that ranged in age from 2 to 19 months. Blood samples were taken from the jugular vein after the morning feeding. Samples were collected into lithium heparin and centrifuged at 1,400 × g for 15 min (at 4°C). Plasma was removed, and the erythrocytes were washed three times with ice-cold 0.15 M saline. The packed erythrocytes were frozen at –80°C and thawed at 26°C. Samples were then diluted 1:8 with distilled water. The hemolysates were centrifuged at 27,000 × g for 30 min (at 4°C), and the stroma was subsequently removed. The hemolysates were stored at –80°C until assayed.

Determination of CA isozyme concentrations: The concentrations of CA-I and CA-II in equine erythrocytes were determined by ELISA. Each well on a flat-bottom micro-ELISA plate was coated by physical adsorption at 4°C for 16 hr with 0.1 m*l* of anti-CA-I or anti-CA-II IgG dissolved in 0.1 M $NaHCO_3$ (pH 8.5). The plates were then washed 4 times with 0.4 m*l* of 0.15 M phosphate-buffered saline (0.9% NaCl) solution containing 0.05% Tween 20 (PBS-Tween). After incubation at 23°C for 2 hr with 0.2 m*l* of 0.5% bovine serum albumin (BSA) in 0.05 M Tris-HCl (pH 8.0), each well was washed 3 times with PBS-Tween. Standard CA isozymes (5 to 800 *n*g/m*l*) or blood samples were diluted with 0.05 M Tris-HCl (pH 7.5) containing 0.3% BSA, 0.9% NaCl, 0.01% thimerosal, and 0.0l M EDTA (buffer A) and subjected to ELISA in duplicate. At the same time, 0.1 m*l* of biotinylated CA-I or biotinylated CA-II diluted with buffer A was added. After incubation at 23°C for 7 hr, each well was washed with PBS-Tween 4 times. Then 0.1 m*l* of the complex of avidin DH and biotinylated horseradish peroxidase H (Vector Laboratories, Inc. Burlingame, Calif. U.S.A.) was added to each well. After 30 min, each well was washed with PBS-Tween 4 times. The peroxidase activity in the wells was measured after adding 0.1 m*l* of ABTS peroxidase substrate (Kirkegaard & Perry Laboratories Inc, Gaithersburg, Calif. U.S.A.). After 10 min, 0.1 m*l* of 1% sodium dodecyl sulfate was added to terminate the reaction. The absorbance at 405 nm was measured with an automatic ELISA reader (ImmunoReader Nj-2000: Japan Inter Med, Tokyo, Japan).

To determine optimum assay conditions, several experiments were performed. First, the microplate was coated with several concentrations of antibody and calibration curves were drawn. A concentration of 10 μg/m*l* of primary antibody and a concentration of 30 *n*g/m*l* of biotinylated CA isozyme were chosen. Assay precision was evaluated with 9 standard samples that were each assayed 5 times in one assay. The coefficients of variation for each assay were less than 5%. To test the sensitivity of the assay, the hemolysate was diluted 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000 and 1:20,000 with buffer A. A linear relationship between estimated CA isozyme and the hemolysate dilution was obtained from 1:4,000 to 1:16,000.

Hemoglobin assay: The hemoglobin concentration in the hemolysate was measured by the sodium lauryl sulfate hemoglobin method in a hemoglobin B test (Wako Pure Chemical Industries Ltd, Tokyo, Japan).

Protein assay: The soluble protein concentration was determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, Calif.).

Statistical analysis: Data analysis was conducted by ANOVA and Fisher's PLSD test. A significance level of $P<0.05$ was chosen.

RESULTS

Specificity of antiserum to CA isozymes: Antibodies to equine CA-I and CA-II were produced in rabbits. The double immunodiffusion test is shown in Fig. 1. Antiserum to equine CA-I and CA-II reacted with equine hemolysate, and the precipitation lines completely crossed.

Measurement of CA-I and CA-II: Typical standard curves for a reference range of CA-I and CA-II solutions (10 to 800 *n*g/m*l*) are shown in Fig. 2. The levels of both CA-I and CA-II in 150 erythrocyte samples from mature thoroughbreds were measured by ELISA. The levels of CA-I and CA-II were expressed as milligrams of enzyme per gram of hemoglobin (Hb). The mean (± SD) concentrations of CA-

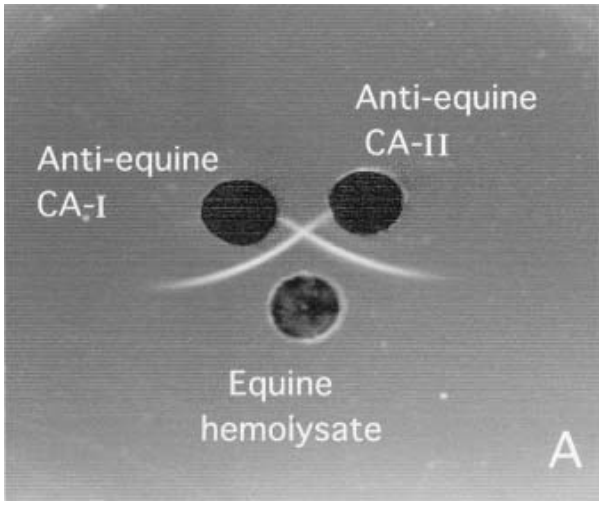

Fig. 1. Result of double immunodiffusion (Ouchterlony test). Notice that the lines of precipitation completely cross for anti-equine CA-I and anti-equine CA-II tested against equine hemolysate.

I and CA-II in erythrocytes of racehorses were 1.70 ± 0.48 and 0.94 ± 0.13 mg/g Hb, respectively. We did not detect significant differences ($p>0.9$) among various age groups (from 2 to 6 years old) and according to sex.

Mean concentrations of CA-I and CA-II in erythrocytes of riding horses were 2.34 ± 0.52 and 0.76 ± 0.08 mg/g Hb, respectively. No significant differences were detected in the concentrations of CA-I or CA-II among male (n=6), female (n=5) and gelding (n=16) riding horses ($p>0.3$). There were no significant differences among CA isozymes in various age groups of racehorses or riding horses ($p>0.9$). When racehorses and riding horses of the same age (from 4 to 6 years) were compared, the CA-I level in riding horses was higher than that in racehorses ($p<0.01$). On the other hand, the CA-II level in racehorses was higher than that of riding horses ($p< 0.02$).

The changes in CA isozyme concentrations with age are shown in Figs. 3A and 3B. The CA-I levels in erythrocytes significantly increased from 2 to 17 months of age. Although not statistically significant, the CA-II levels slightly decreased with age (Fig. 3B).

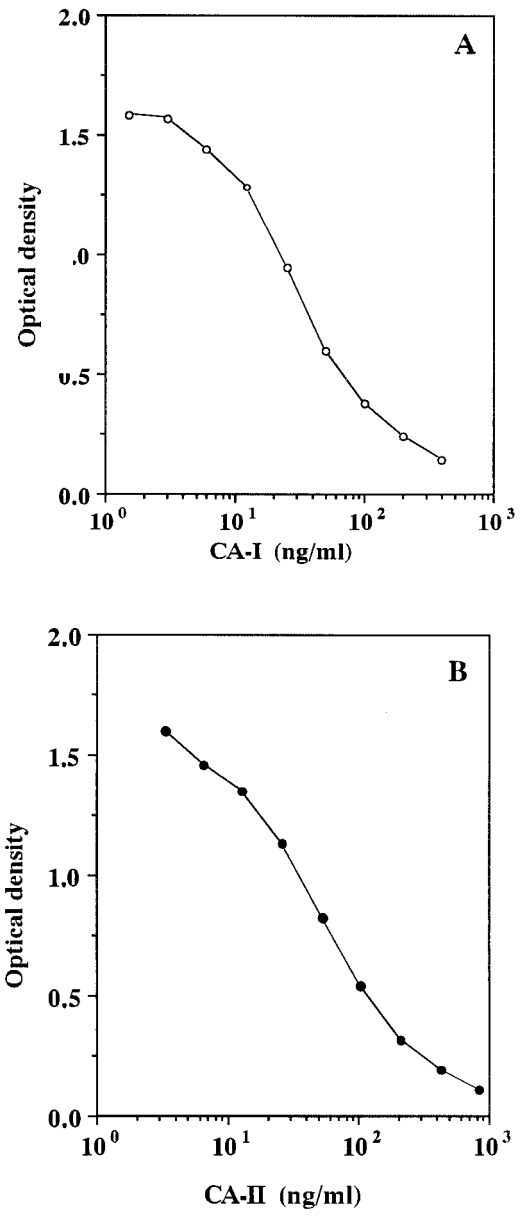

Fig. 2. Standard curve for equine CA-I (top) and CA-II (bottom) concentrations determined by ELISA. Values were obtained from a spectroscope at 405 nm.

DISCUSSION

Separate measurements of the CO_2 hydrase activity of CA-I and CA-II require specific inhibitors or separation of the isozymes. It is difficult to study the factors and conditions that affect CA activity, because standard CA activity assays have serious limitations. Therefore, estimates of the CA-I and CA-II levels in erythrocytes are complicated by the pronounced differences in enzymatic activities of CA-I and CA-II. In most mammalian species, CA-I and CA-II isozymes can be immunologically differentiated. In the present study, equine CA-I and CA-II were clearly differentiated with anti-equine CA-I and anti-equine CA-II antisera. For these reasons, immunologic analysis is superior to kinetic assays for determining the concentrations of CA-I and CA-II in erythrocytes. Many samples can be simultaneously tested with ELISA, which is quicker and more sensitive than the passive hemagglutination inhibition technique or single radial immunodiffusion. The detection limit for single radial immunodiffusion is 0.01 to 1 mg/m*l* of human CA-I [8]. Furthermore, biotin-labeled reagents are cheap, safe and stable and have a long shelf life. The ELISA described in this study is a precise and sensitive assay for equine CA-I and CA-II.

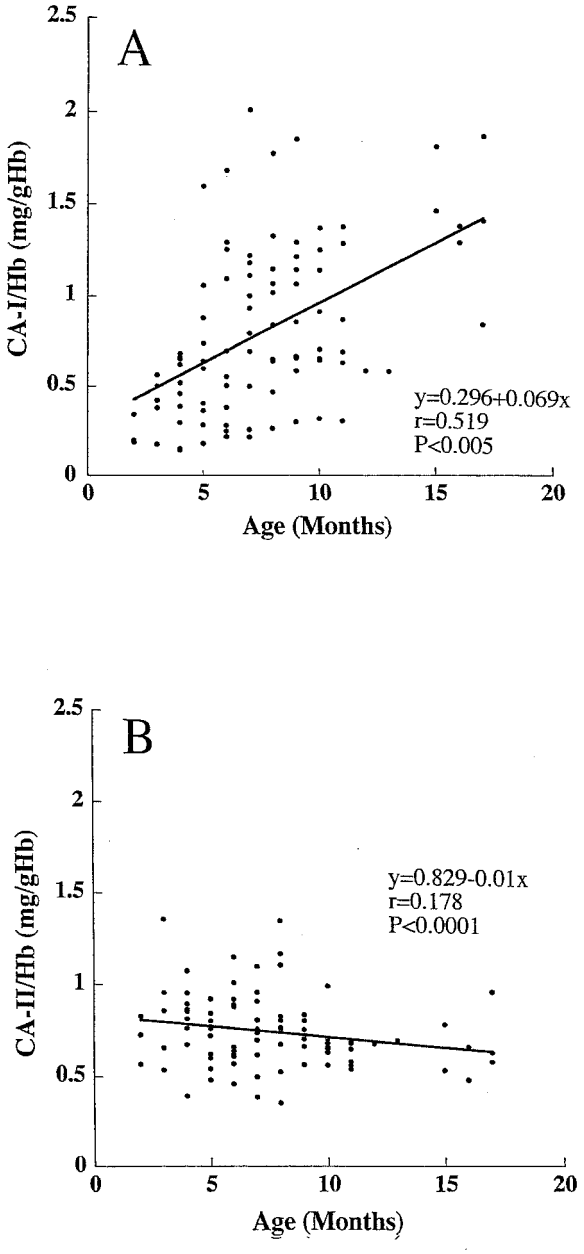

Fig. 3. Correlation between age and concentration of CA-I (top) and CA-II (bottom) in immature horses between 2 and 17 months old.

CA-I and CA-II are both found in the erythrocytes of most mammalian species. Ruminants are an exception, as they express only the CA-II isozyme [3] and the concentration of CA-II in bovine erythrocytes is 14.22 mg/g Hb (unpublished data). Mean concentrations of CA-I and CA-II in erythrocytes from thoroughbreds, Beagles [12], dogs native to Japan [12] and humans [1] are 1.89 and 0.89 mg/g Hb, 3.21 and 1.63 mg/g Hb, 11.03 and 3.29 mg/g Hb, and 13.68 and 1.59 mg/g Hb, respectively. Among these animals there is a large difference in CA-I levels in erythrocytes; nevertheless, with the exception of cows, the difference in CA-II levels is small. These data suggest that CA-I levels in erythrocytes are vary according to the species.

Although carbonic anhydrase levels in erythrocytes vary little in a healthy human population, a wide variation occurs in various disease states [8]. And, the levels of CA-I and CA-II in maternal erythrocytes at parturition are almost 20% greater than normal levels [9]. It has been suggested that CA-II is physiologically more important than CA-I in erythrocytes and renal tubules [17]. Villeval *et al.* [19] reported that CA-I was an early specific marker of normal erythroid differentiation in humans. Aliakbar and Brown [1] reported that the levels of human CA-I and CA-II in neonatal blood were 8.8% and 28.8%, respectively, of the levels in adult blood.

In the present study, the erythrocyte levels of CA-I and CA-II in 2-month-old horses were about 13% and 90%, respectively, of the levels in mature horses. The equine CA-I level increased with age, but the equine CA-II level decreased slightly with age so that, the expression of CA-I and CA-II is regulated differently during development. Shepherd *et al.* [16] reported that the level of CA-I in human erythrocytes increased noticeably during this first year of life. These human CA-I data are similar to the equine CA-I data in the present study. In one horse that we studied, the CA-I level in erythrocytes did not substantially increase with age. At 3 months of age, the CA-I concentration was 0.17 mg/g Hb. By 15 months of age, the CA-I level was only 0.54 mg/g Hb, which is 30% of the CA-I level in healthy 15-month-old horses. The CA-II level in this horse was similar to those of healthy horses. Although the average body weight of a 6-month-old horse is 350 kg, this horse weighed only 300 kg at 6 months. Eventually this horse was euthanized.

Ohno *et al.* [13] reported that the CA-I levels in human erythrocytes decreased by about 20% immediately after 30 min of physical exercise. The CA-I levels returned to the pre-exercise levels after 30 min of rest. The CA-II levels were unaffected by recent exercise. In the present study, recent exercise did not affect the levels of CA-I, because the blood samples were collected from resting horses, but the CA-I levels of racehorses were about 28% less than those of riding horses. In contrast, the levels of CA-II in racehorses were about 20% greater than those of riding horses.

The level of CA-II in a 17-month-old horse was similar to those of adult riding horses. The CA-II levels in the erythrocytes of racehorses were probably increased because of intensive daily exercise. These data suggested that the levels of CA isozymes in erythrocytes of racehorses were influenced by chronic physical stress.

The method developed in the present study will enable investigators to evaluate changes in isozymes associated with development and hereditary or metabolic disorders. CA isozyme concentrations can be measured in a small sample of blood or other body fluids. Additional studies are

needed to clarify the clinical usefulness of equine CA-I and CA-II concentrations.

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