In Vitro and *in Vivo* Effects of Dantrolene on Carbonic Anhydrase Enzyme Activities

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The effects of dantrolene were investigated on carbonic anhydrase (CA) enzyme activities in *in vitro* human and in *in vivo* Sprague-Dawley rat erythrocytes. For *in vitro* study, human carbonic anhydrase-I (HCA-I) and -II (HCA-II) were purified by Sepharose 4B–L-tyrosine-sulfanylamide affinity chromatography, rats were used for *in vivo* study. *In vivo* and *in vitro* CA enzyme activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson. Dantrolene (1.64×10^{-5}—6.56×10^{-5} M) showed *in vitro* inhibitory effects on HCA-I and HCA-II hydratase activity, when determined using the CO_2-hydratase method. 50% inhibitory concentration (IC_{50}) was 4.09×10^{-5} M for HCA-I and 3.24×10^{-5} M for HCA-II. Rat erythrocyte CA activity was significantly inhibited by 10 mg/kg dantrolene for up to 3 h ($p<0.001$) following intraperitoneal administration. In conclusion, Dantrolene inhibited the carbonic anhydrase enzyme activity under *in vitro* and *in vivo* conditions.

Key words dantrolene; carbonic anhydrase; erythrocyte; human; rat

Carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) is a well-characterized pH regulatory enzyme in most tissues including erythrocytes, and catalyzes reversible hydration of CO_2 to HCO_3^- and H^+. The only known physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3^-, they therefore play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis.[1)] Up to now, fourteen different CA isozymes have been described in higher vertebrates.[2)] Among CA isozymes are the cytosolic ones (CA I, CA II, CA III, CA VII); CA-I is found together with CA-II in erythrocytes. CA-II is the most widely distributed CA in the eye, kidney, central nervous system (CNS) and inner ear. There are also membrane-bound (CA IV, CA IX, CA XII, CA XIV), mitochondrial (CA V), secretory forms (CA VI) and several acatalytic forms (CA VIII, CA X, CA XI).[3—5)]

CA plays an important role in water and ion transport and pH regulation in kidney, eye, CNS, inner ear and other systems. The CA inhibitor acetazolamide is used for diuresis in some disorders. It may be said that CA plays a role in endolymph and cerebrospinal fluid synthesis and pH regulation, due to the beneficial effects of acetazolamide on endolymphatic hydrops or hydrocephalus.[6)] The activity of CA isozymes in human erythrocytes has been shown to vary considerably under physiological conditions. Moreover, changes in CA activity have been associated with such metabolic diseases diabetes mellitus and hypertension.[7,8)] The inhibition of its has been shown to impair H^+ secretion into the proximal small intestinal lumen, thereby decreasing bicarbonate resorption. In addition, inhibition of CA leads to decreased acidification of urine, the production of alkaline urine and eventually metabolic acidosis.[9)]

Hydantoin derivative dantrolene (1-[[[5-(4-nitrophenyl)-2-furanyl]methylene]amino]-2,4-imidazolidine-dione sodium salt hydrate) is a peripherally acting skeletal muscle relaxant, and is clinically used in the treatment of muscle spasticity, malignant hyperthermia, and neuroleptic malignant syndrome.[10)] It depresses excitation–contraction coupling in the muscle fibre by inhibiting calcium release from the sarcoplasmic reticulum.[11)] It also shows calcium channel blocker effect in the smooth muscle membrane, antioxidative and cytoprotective properties.[12—18)]

Although dantrolene is used in the treatment of muscle spasticity, malignant hyperthermia, and neuroleptic malignant syndrome, its impact on carbonic anhydrase (CA) activity has not previously been reported. In the present study, the effects of dantrolene were investigated on carbonic anhydrase enzyme activity in *in vitro* human and *in vivo* rat erythrocytes.

MATERIALS AND METHODS

Materials Sepharose 4B, protein assay reagents, chemicals for electrophoresis and dantrolene were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from 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.

Purification of HCA-I and HCA-II by Affinity Chromatography Erythrocytes were purified from fresh human blood (10 ml), which was obtained from the University Hospital Blood Center. Following low-speed centrifugation (1500 rpm for 15 min) (Heraeus Sepatech, Suprafuge 22) and removal of plasma and buffy coat, 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 then removed by high-speed centrifugation (20000 rpm for 30 min) (Heraeus Sepatech, Suprafuge 22) at 4 °C and the pH of the haemolysate adjusted to 8.7 with solid Tris. The pH-adjusted haemolysate was then subjected to affinity chromatography [*Chromatography cystem*: 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 isozymes. Haemoglobin (Hb) concentration in haemolysate was determined by cyanmethemoglobin method.[19)]

One hundred eighty milliliters of pH-adjusted human erythrocyte haemolysate was applied to the Sepharose 4B–L-ty-

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rosine-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, 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 isozyme purification procedures, 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.[20,21]

Measurement of CA Activity CA activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[20] CO_2-hydratase activity of enzyme was determined at room temperature in a veronal buffer (pH: 8.6) with Bromo Timol Blue as indicator and satured carbon dioxide solution as substrate in a final volume of 4.2 ml. The time (in seconds) taken for the solution to change from blue to yellow was measured. The enzyme unit (EU) was calculated 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.

***In Vitro* Inhibition Studies** The effect of increasing concentrations of dantrolene (1.64×10^{-5}—6.56×10^{-5} M) on human CA isozyme activity was determined calorimetrically using the CO_2-hydration method of Wilbur and Anderson.[20] The mathematical relationship between dantrolene concentration and CA activity was determined using conventional polynominal regression software (Microsoft Office 98, Excel).

In Vivo* Inhibition Studies** Eight adult Sprague-Dawley rats with a weight of 200—250 g were used for the experiment. The animals were fed standard laboratory chow and tap water before the experiment. Before dantrolene administration, a 0.5 ml blood sample was taken from a tail-vein, then 10 mg·kg^{-1} of dantrolene was intraperitoneally administered. At 1, 3 and 6 h after dantrolene administration, 0.5 ml blood samples were taken again. All blood samples were placed in test tubes with EDTA (2 mM) and subjected to centrifugation at 2500×g*** for 15 min at 4 °C (HERMLE Z383K). The erythrocyte pellet was washed three times with cold 0.16 M KCl and the supernatant discarded. One volume of erythrocyte pellet was suspended in five volumes of ice water to give an erythrocyte haemolysate. Carbonic anhydrase activity was determined colorimetrically as described above.[20,21] Data were expressed as the mean±S.D. (standard deviation). Statistical analysis comprised significance testing of the difference between means (control *vs.* test) using a two-tailed Student's *t*-test at the levels: 0.05, 0.01, and 0.001.

Protein Determination Quantitative protein determination was done by absorbance measurement at 595 nm according to Bradford, with bovine serum albumin as standard.[24]

SDS Polyacrylamide Gel Electrophoresis Discontinuous polyacrylamide gel electrophoresis has performed under denaturing conditions after HCA-I and HCA-II purification according to the method of Laemmli.[25] For HCA-I 20, 30 and 40 μg samples were applied to the electrophoresis medium in line 1, 2, 3, respectively. For HCA-II 20, 30 and 40 μg samples were applied to the electrophoresis medium in line 4, 5, 6, respectively. Gel was stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained by frequently changing the same solvent, without dye. The electrophoretic pattern was photographed (see Fig. 1).

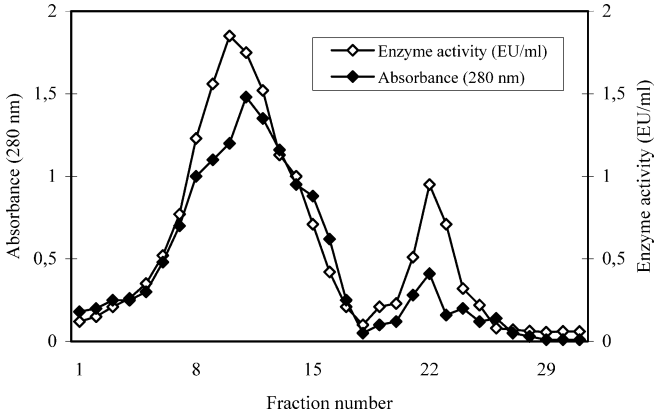

Fig. 1. Elution Graph of HCA-I and HCA-II Isozymes from Human Erythrocytes with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), Respectively

Flow rate: 20 ml h^{-1}, fraction volume: 4 ml.

RESULTS AND DISCUSSION

It is generally recognized that CA 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.[26] Given the physiological importance of CA, the metabolic impact of medically important drugs should receive greater study, not only erythrocyte HCA-I and HCA-II but also whole CA isozymes. For example, in two recent studies, total hepatic CA (I+II+III+IV) activity was shown to diminish in the streptozotocin-induced diabetic rat.[27] Gluconeogenesis and ureagenesis were also associated with an increase in hepatic CA-V activity.[28] In addition, hepatic pH disequilibrium was explained in terms of changes in CA activity. Furthermore, many drug side effects are believed to result from CA isozyme inhibition. For example, respiratory acidosis is probably the cause of some side effects observed during acetazolamide therapy, such as fatigue, headache, altered taste sensations and distress.[29]

The effects of increasing concentration of dantrolene administration on human erythrocyte HCA-I and HCA-II isozymes were undertaken in this study. Thus, HCA-I and HCA-II were purified by Sepharose 4B–L-tyrosine-sulfanylamide affinity chromatography (Table 1, Fig. 1) and the purity confirmed by SDS-PAGE (Fig. 2). The overall purification of HCA-I and HCA-II isozymes was obtained with a yield of 60.8, 34.5, and a specific activity of 5955.5, 18143 U/mg proteins, and these enzymes were purified 681, 2073-fold, respectively (Table 1). The range of dantrolene concentrations used was considered adequate to show enzyme inhibition effects. In this regard, it was evident from *in vitro* studies that the HCA-I and HCA-II were inhibited by dantrolene at 1.64×10^{-2}—6.56×10^{-2} mM concentrations (Fig. 3). Fifty percent inhibitory concentration (IC_{50}) was 4.09×10^{-5} M for HCA-I (r^2: 0.9979) and 3.24×10^{-5} M for

Table 1. Purification Scheme of HCA-I and HCA-II from Human Erythrocytes by Sepharose-4B–L-Tyrosine-sulfanilamide Affinity Chromatography

Purification step		Activity (U/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Spesific activity (U/mg)	Yield (%)	Purification factor
Haemolysate		980	180	112	20160	176400	8.75	100	1
Sepharose-4B–L-tyrosine-sulfanilamide affinity chromatography	HCA-I	5360	20	0.90	18.0	107200	5955.5	60.8	681
	HCA-II	3810	16	0.21	3.36	60960	18143	34.5	2073

Experimental analyses were performed at +4 °C. CA enzyme activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson. The enzyme unit (EU) was calculated 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.

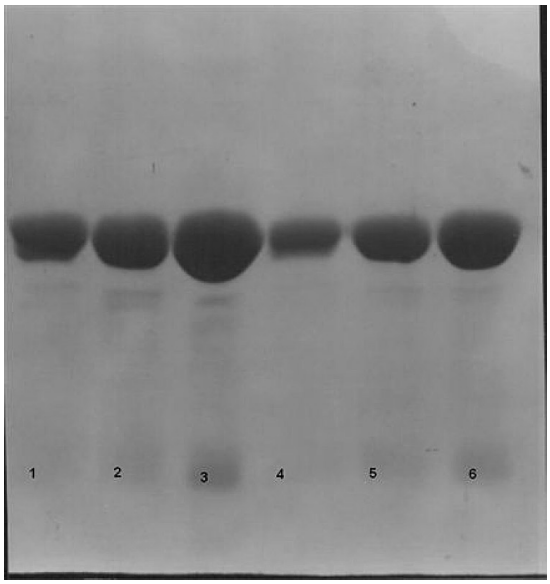

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of HCA-I and HCA-II are Purified by Sepharose 4B–L-Tyrosine-sulfanylamide Affinity Gel Chromatography

1, 2 and 3 lanes are HCA-I, 4, 5 and 6 lanes are HCA-II.

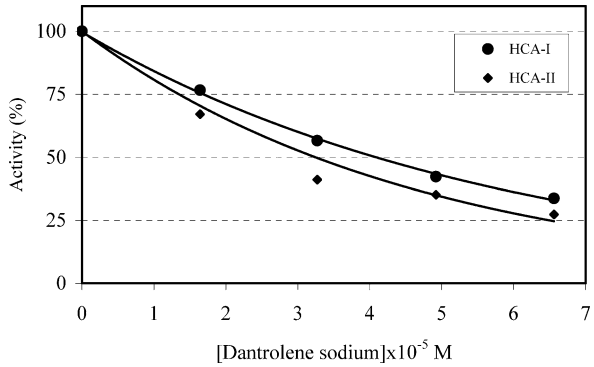

Fig. 3. Effect of Dantrolene Concentrations (4.09×10^{-5} M for HCA-I and 3.24×10^{-5} M) on Human Erythrocyte CA-I and CA-II Activities

Experimental analyses were performed at +4 °C. CA enzyme activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson. The enzyme unit (EU) was calculated 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.

HCA-II (r^2: 0.9547). In *in vivo* study, maximal inhibition of rat erythrocyte CA enzyme activity by 10 mg·kg^{-1} dantrolene occured within 1 h ($p<0.001$; Table 2) after intraperitoneal drug administration and inhibition significantly continued even after 3 h ($p<0.001$; Table 2). The half-life of dantrolene is 31 min in rat plasma. In our study, maximal inhibition of CA enzyme activity was seen within 1 h after drug administration and significant inhibition continued even after 3 h (Table 2). These results showed that there is a correlation between the plasma half-life of dantrolene and inhibition of CA enzyme activity.[30] Our data showed that there is accordance between *in vivo* and *in vitro* effects of dantrolene on CA activity.

Table 2. Effect of Dantrolene (10 mg·kg^{-1}, i.p.) on Rat Carbonic Anhydrase Activity

Time after administration (h)	Carbonic anhydrase activity (EU/gHb)[a,b]
0	58.518±1.943
1	42.428±1.383*
3	45.857±2.416*
6	56.940±2.867

Haemoglobin (Hb) concentration in haemolysate was determined by cyanmethemoglobin method. Experimental analyses were performed at +4 °C. CA enzyme activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson. The enzyme unit (EU) was calculated 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. *a*) Results are expressed as the mean±standard deviation, $n=8$. *b*) $*p<0.05$, *vs.* control by Student's *t* test. (Control value is enzyme activity before dantrolene administration; 0 h) were analyzed.

CA is present in a number of extrarenal tissues including eye, gastric mucosa, central nervous system and erythrocytes. Inhibition of CA in the ciliary processes of the eye decreases the rate of formation of aqueous humor and reduces intraocular pressure. Due to interference with CA activity in RBCs, CA inhibitors increase CO_2 levels in peripheral tissues and decrease the amount of CO_2 in expired gas; therefore, acidosis may appear.[27] On the other hand, large doses of CA inhibitors reduce gastric acid secretion.[27] CA inhibitors are used for treatment of edema, glaucoma or epilepsy. Acute mountain sickness is the most common and may appear at altitudes as low as 2000 m[31]; acetazolamide is an effective prophylactic for this condition. The mechanism for the beneficial effect of acetazolamide in acute mountain sickness is not clear, but it may be related to the induction of a metabolic acidosis.[27] The *in vivo* inhibitory effect on erythrocyte CA activity and associated sequelae should be considered in the therapeutic application of dantrolene. Due to the CA enzyme inhibitor effect, dantrolene may be useful for correcting a metabolic alkalosis, edema, glaucoma, epilepsy or acute mountain sickness. In a previous study it was shown that dantrolene has antiulcerogenic property; this effect may

be partly related to the inhibition of CA enzyme activity in stomach tissue.[14)]

In conclusion, dantrolene showed *in vitro* and *in vivo* inhibition effects on erythrocyte HCA-I and HCA-II activities. It can be useful for correcting a metabolic alkalosis, edema, glaucoma, epilepsy or acute mountain sickness. The use of dantrolene in a patient with metabolic acidosis can cause serious side effects and worsen the health of the patient. For this reason, dantrolene should be carefully used and the dosage should be very well ordered to minimize side effects.

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