Morphine Inhibits Erythrocyte Carbonic Anhydrase *in Vitro* and *in Vivo*

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Morphine is implicated in diverse functions, from development to immune modulation in the central and peripheral nervous systems. At the present time, morphine is one of the most effective antinociceptive agents used to manage pain. It has been used extensively in the clinical management of pain due to its potent analgesic effect. In this study, the *in vitro* and *in vivo* inhibitory effects of morphine on erythrocyte carbonic anhydrase (CA) were investigated. Human erythrocyte isoenzymes, HCA-I and HCA-II, were purified by Sepharose-4B affinity chromatography column with a yield of 66.95 and 62.82%, a specific activity of 3892.3 and 11663.2 EU/mg proteins with 745.1 and 2232.6-fold purification of each isoenzyme, respectively. To determine enzyme purity, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. *In vitro* inhibition of erythrocyte HCA-I and HCA-II by morphine using the CO_2-hydratase enzyme gave IC_{50} values 4.50×10^{-5} M (r^2: 0.954) and 9.23×10^{-5} M (r^2: 0.996), respectively. CA activity was significantly attenuated *in vivo* in Spraque-Dawley rats for up to 3 h ($p<0.001$) following intraperitoneal administration of morphine. In conclusion, morphine inhibited CA activity both *in vitro* and *in vivo*.

Key words carbonic anhydrase; morphine; erythrocyte; inhibition; enzyme

Carbonic anhydrase (CA; Carbonate hydrolyase, EC 4.2.1.1) is a family of metalloenzymes that catalyze the rapid conversion of carbon dioxide to bicarbonate and protons and involved in the biomineralization process.[1)] In the biomineralization process, the mineral structures involved are mainly calcium carbonate and calcium phosphate crystals, in invertebrates and vertebrates, respectively. This enzyme is a multifunctional enzyme that catalyzes the hydration/dehydration of carbon dioxide. The molecular characteristics of the CA across the plant and animal kingdoms are similar.[2)] It catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+. In the red blood cell, this enzyme is necessary to facilitate the transport of carbon dioxide out of the body.[3)]

$$CO_2+H_2O \rightleftarrows H_2CO_3^- \rightleftarrows H^+ + HCO_3^-$$

Carbonic anhydrase greatly increases the rate of the reaction, with typical catalytic rates of the different forms of this enzyme ranging between 10^4 and 10^6 reactions per second. The active site of most carbonic anhydrases contains a zinc ion.[4)]

Many natural and synthetic substances can affect the living metabolism by altering enzyme activities and affecting metabolic pathways at low concentrations.[5)] For instance, gentamisin sulphate inhibits human CA-I and CA-II isozyme activity.[5)] In addition, melatonin and dantrolene which are used in medical treatment have been shown to inhibit erythrocyte HCA-I and HCA-II activity.[3,6)] CA plays an important role in water and ion transport and pH regulation in kidney, eye, central nervous system (CNS), inner ear and other systems. Morphine is a phenanthrene opioid receptor agonist. The main effect of morphine is binding to and activating the μ-opioid receptors in the CNS. In this study, CA-I and CA-II isozymes, which has a vital importance in living metabolism, was purified at only one step *via* Sepharose 4B-L tyrosine affinity column from human erythrocytes. The main objective of this research was to determined *in vitro* and *in vivo* effects of morphine on CA-I and CA-II isozymes.

MATERIALS AND METHODS

Chemicals CNBr activated-Sepharose 4B, protein assay reagents and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. L-Tyrosine and *para*-aminobenzene sulfonamide were from Merck (Germany). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck. Morphine was provided from the Department of Pharmacy, Atatürk University Research Hospital, Erzurum, Turkey.

Purification of Human Erythrocyte Carbonic Anhydrase-I and II Isozymes Erythrocytes were isolated from fresh human blood, which was obtained from the University Hospital Blood Centre following low-speed centrifugation at 1500 rpm for 15 min (MSE, MISTRAL 2000) by removal of plasma and buffy coat. The red cells were 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 at 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 using a column packed with Sepharose 4B-L-tyrosine sulphanilamide resin to a bed volume of 25 ml and pre-equilibrated with 25 mM Tris/HCl/0.1 M Na_2SO_4, pH 8.7.[7)]

One hundred milliliters of pH-adjusted human erythrocyte haemolysate was applied to the column and 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 sodium phosphate; pH 6.3 and 0.1 M sodium acetate/0.5 M $NaClO_4$; pH 5.6, respectively. Column flow rate was 20 ml/h and was 4 ml fractions were collected in a fraction collector (AO Instrument Co.) at 4 °C

Absorbance 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.[8,9)]

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Protein Determination Quantitative protein determination was done by absorbance measurement at 595 nm after reaction with Bradford reagent using bovine serum albumin as standard.[9]

SDS polyacrylamide Gel Electrophoresis Discontinuous polyacrylamide gel electrophoresis was performed under denaturing conditions after HCA-I, HCA-II purification, according to the Laemmli's procedure.[10] The stacking and running gels comprised 3% (w/v) and 10% (w/v) acrylamide, respectively, and 0.1% (w/v) sodium dodecyl sulphate (SDS). The electrode buffer was 0.025 M Tris/0.2 M glycine, pH 8.3.

The sample buffer was prepared by mixing 0.65 ml of 1 M Tris–HCl, pH 6.8, 3 ml of 10% (w/v) SDS, 1 ml of neat glycerol, 1 ml of 0.1% (w/v) bromphenol blue, 0.5 ml β-mercaptoethanol and 3.85 ml water. A 20 μg aliquot of enzyme (50 μl) was added into 50 μl sample buffer and the mixture was heated in a boiling water bath for 3 min and cooled.

The HCA-I and HCA-II isozyme samples were loaded into each space of the stacking gel (slab gel dimensions, 16×18 cm). Rat CA was analyzed separately by polyacrylamide gel electrophoresis. Initially, an electric potential of 80 V was (Hoefer Scientific Instruments SE 600) applied until the bromphenol dye reached the running gel. Then it was increased to 200 V for 3—4 h. Gels were stained for 1.5 h in 0.1% (w/v) Coommassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid, and distained with methanol/acetic acid. The electrophoretic pattern was photographed (see Fig. 1).

***In Vitro* Inhibition Studies** The effect of increasing concentrations of morphine (1.6×10^{-5}—19.2×10^{-5} M) on HCA isozyme activity was determined colorimetrically using CO_2-hydration method of Wilbur and Anderson[16] modified by Rickli and co-workers.[8] Briefly, the enzyme sample was added to 4.2 ml (final volume) of incubation mixture containing 1 ml 0.025 M veronal buffer (pH 8.2) +0.1 ml 0.04% (w/v) bromthymol blue, and 2.5 ml saturated CO_2 solution in water. Enzyme activity was measured by colorimetric method considering the time necessary to change the pH from 8.2 to 6.3. One enzyme unit (EU) of CO_2-hydratase activity was calculated by using the equation $(t_o-t_c)/t_o$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

The mathematical relationship between morphine concentration and CA activity (expressed as a percentage of that in the absence of morphine) was determined using conventional polynomial regression software (Microsoft Office 97, Excel).

In Vivo* Inhibition Studies** Eight adult Sprague-Dawley rats (200—250 g) were selected for intraperitoneal administration of morphine (5 mg/kg body weight). Blood samples (10×0.5 ml) were taken from each rat prior to morphine administration as well as at 1, 3 and 5 h intervals thereafter. The blood was placed into test tubes containing 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. CA activity was determined colorimetrically as described above.[7,8]

Statistical Analysis All data were expressed as the mean±S.D. A two-tailed Student's *t*-test was used for significance testing of the difference between means (control *vs.* test) at the 0.05, 0.01 and 0.001 levels.

RESULTS AND DISCUSSION

Morphine is widely used for the management of pain; its clinical usefulness is partially limited, especially in non-cancer pain patients, by the development of tolerance and dependence that occurs after repeated treatment. Antinociceptive morphine tolerance is indicated by a decreased efficacy of the drug with repeated administration and results in a need to increase the morphine dose in order to achieve the desired analgesic effect. However, the possibility of using increasing doses of morphine is limited by the occurrence of adverse side effects such as respiratory depression and constipation.[11,12]

The various CA isozymes are found in many different tissues. They are involved in a number of different physiological processes, including bone resorption, calcification, ion transport, acid-base transport, and a number of different metabolic processes. In mammals, CA is expressed in almost all tissues. Proposed functions include oxygen transport between lungs, red blood cells and tissues, pH regulation, ion exchange in the kidney, and electrical activity in the retina and nervous system.[13—15] Another situation in which the role of CA has been extensively investigated is in autotrophic organisms, where CA is mostly related to the provision of bicarbonate for carbon fixation.[16,17]

Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme.[18] The effects can be dramatic and systemic.[19] Indeed, CA isozymes are important enzymes in metabolism because they regulate pH in most tissues. CA inhibitors vary according to their affinity of binding to a particular CA isozyme, potency for inhibiting that isozyme and physicochemical properties, which can influence their tissue distribution and scope of activity.[20]

In recent studies, it was shown that CA was potently inhibited by several members of sulphamate-containing drugs.[21] 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 the entire array of 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.[22] Gluconeogenesis and ureagenesis were also associated with an increase in hepatic CA-V activity.[23] There is convincing evidence for over expression of CA-IX and CA-XII in cancer,[24] acidifying the extracellular matrix, which is thought to promote growth of the tumour. Solid tumours are often hypoxic, and expression of CA-IX and CA-XII is upregulated under these conditions. CA present within the ciliary epithelium is associated with aqueous humour production and intraocular pressure. It is important for retina metabolism, acid-base homeostasis, and synaptic transmission.[25] In addition, hepatic pH disequilibrium was explained in terms of changes in CA activity. Furthermore, many drug side effects may be considered 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.[22]

Measurement of the CO_2 hydratase activity of CA-I and CA-

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)	Specific activity (U/mg)	Yield (%)	Purification factor
Haemolysate		512	124	98	12152	63488	5.224	100	1
Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography	HCA-I	2024	21	0.52	10.92	42504	3892.3	66.95	745.1
	HCA-II	2216	18	0.19	3.60	39888	11663.2	62.82	2232.6

HCA: human carbonic anhydrase. Purification procedures were performed at +4 °C. Enzyme activity was determined colorimetrically using CO2-hydration method of Wilbur and Anderson. 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.

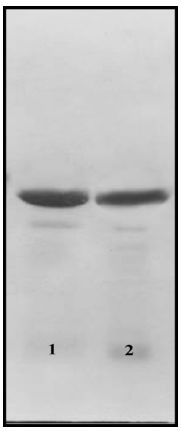

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis of HCA-I and HCA-II Purified by Sepharose 4B-L-Tyrosine-Sulphanilamide Affinity Gel Chromatography

HCA: human carbonic anhydrase, lane 1 is HCA-I, lane 2 is HCA-II.

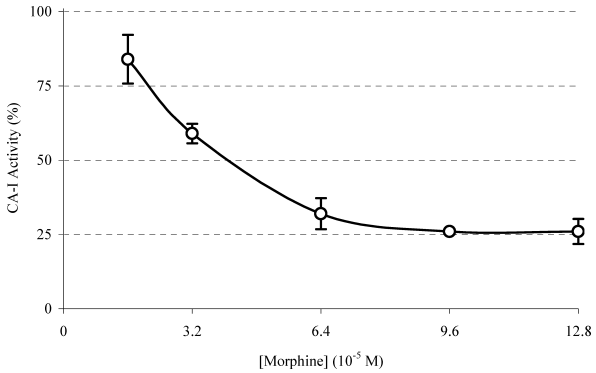

Fig. 2. Effect of Morphine at Different Concentrations on Human Erythrocyte CA-I Activity

CA-I: carbonic anhydrase isoforms-I. Each value is average of three independent experiments.

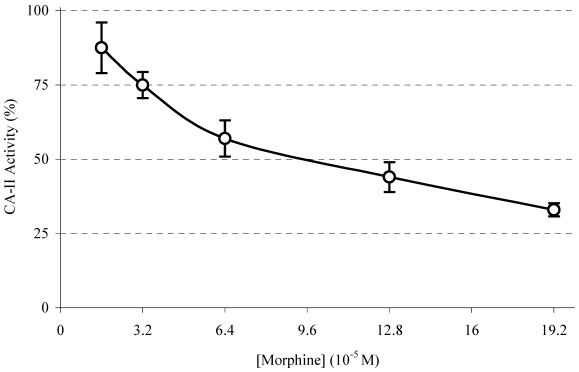

Fig. 3. Effect of Morphine at Different Concentrations on Human Erythrocyte CA-II Activity

CA-II: carbonic anhydrase isoforms-II. Each value is average of three independent experiments.

II requires 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, estimate of the CA-I and CA-II level in erythrocytes are complicated by the pronounced differences in enzymatic activity of CA-I and CA-II.[26)]

Given the importance of CA in pH regulation in most tissues, the effects of increasing concentration of morphine 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). As can be seen Fig. 1, the purity of both isoenzymes confirmed by SDS-PAGE. The overall purification, HCA-I and HCA-II isozymes were obtained with a yield of 66.95, 62.82 and a specific activity of 3892.3, 11663.2 U/mg proteins. Also, these isoenzymes were purified 745.1, 2232.6-fold, respectively (Table 1).

The range of morphine concentrations (1.6×10^{-5}—19.2×10^{-5} M) 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 morphine at 1.6×10^{-5}—19.2×10^{-5} M concentrations (Figs. 2, 3). 50% inhibitory concentrations (IC_{50}) were 4.50×10^{-5} M (r^2: 0.954) for HCA-I and 9.23×10^{-5} M (r^2: 0.996) for HCA-II. In *in vivo* study, maximal inhibition of rat erythrocyte CA enzyme activity by pharmacological dosage (5 mg/kg) of morphine was within 3 h ($p<0.001$; Table 2) after intraperitoneally drug administration. Our data showed that there is

Table 2. The *in Vivo* Effects of Pharmacological Dosage of Morphine (5 mg/kg Body Weight) on Rat Red Blood Cell CA Activity

Time after administration (h)	Total CA activity (%)[a,b)]
0	100.00±4.13
1	86.20±4.81*
3	4.39±0.63**
5	47.41±3.38**

CA: carbonic anhydrase, $n=8$. *a*) Results are expressed as the mean±standard deviation, $n=8$. *b*) $*p<0.05$, $**p<0.01$, *vs.* control by Student's *t* test analysis. Control value is enzyme activity before morphine administration at 0 h.

accordance between *in vivo* and *in vitro* effects of morphine on CA activity. Recently, in another study, IC_{50} values for dantrolene sodium were found as 4.09×10^{-5} M for HCA-I

and 3.24×10^{-5} M for HCA-II. However, maximal inhibition of rat erythrocyte CA enzyme activity by 10 mg/kg dantrolene occurred within 1 h ($p<0.001$).[27] In the same way, HCA-I and HCA-II were inhibited up to 2×10^{-4} M. Conversely, there is no correlation between inhibition of both CA isoenzymes and the process occurring in a dose-dependent manner. After 4×10^{-4} M concentration of melatonin, the inhibitory effects of melatonin decreased with increased melatonin concentration.[3]

CA is present in a number of extrarenal tissues including eye, gastric mucosa, CNS and erythrocytes. Inhibition of CA in the ciliary processes of the eye decreases the rate of formation of aqueous humour and reduces intraocular pressure. Due to interference with CA activity in erythrocytes, CA inhibitors increase CO_2 levels in peripheral tissues and decrease amount of CO_2 in expired gas; therefore, acidosis may appear. On the other hand, large doses of CA inhibitors reduce gastric acid secretion.[28] CA inhibitors are used for treatment of oedema, glaucoma or epilepsy. Acute mountain sickness is the most common and may appear at altitudes as low as 2000 m.[29] Acetazolamide is an effective prophylactic for acute mountain sickness. 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.[28] The *in vivo* inhibitory effect on erythrocyte CA activity and associated sequelae should be considered in the therapeutic application of morphine. Due to its CA enzyme inhibitor effect, morphine can be useful for correcting a metabolic alkalosis, oedema, glaucoma, epilepsy or acute mountain sickness.

CONCLUSION

Since this enzyme produces and uses protons and bicarbonate ions, CA plays a key role in the regulation of pH and fluid balance in different parts of the human body. Especially, in stomach lining it plays a role in secreting acid, while the same enzyme helps to make pancreatic juices alkaline and saliva neutral. The transport of the protons and bicarbonate ions produced in kidney and eyes influence the water content of the cells at these locations. Thus CA isozymes perform different functions at their specific locations. On the other hand, their absence or malfunction can lead to diseased states, ranging from the loss of acid production in the stomach to kidney failure. When there is a build up of fluid that maintains the shape of eyes, the fluid often presses on the optic nerve in the eye and may damage it. This condition is called glaucoma. In recent years, inhibitors of CA are being used to treat glaucoma. Blocking this enzyme shifts the fluid balance in the eyes of the patient to reduce fluid build up thereby relieving pressure. The CA inhibitor sulphonamide is bound to HCA. Note that this inhibitor binds near the active site and disrupts the interactions of the water bound to the zinc ion, blocking the enzyme action. Unfortunately, prolonged use of these inhibitors can affect the same enzyme present in other tissues and lead to side effects like kidney and liver damage.

In conclusion, morphine showed *in vitro* inhibition of erythrocyte HCA-I and HCA-II activities as well as *in vivo* inhibition of rat erythrocyte CA. CA inhibitors can cause of some side effects mentioned above. In this circumstance, the use of morphine in a patient with metabolic acidosis and other complications can cause serious side effects and worsen health of the patient. For this reason, morphine must be carefully used and their dosages should be very well ordered to decrease the side effects. Furthermore, morphine has some side effects like kidney and liver damage or metabolic acidosis.[30] There is any possibility that the side effects of morphine like kidney and liver damage or metabolic acidosis is due to morphine-induced inhibition of CA.

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