Effects of Gentamicin Sulfate on Enzyme Activities of Carbonic Anhydrase from Human Erythrocytes in Vitro and from Rat Erythrocytes in Vivo

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The effects of gentamicin sulfate on carbonic anhydrase (CA) enzyme activity in in vitro human and in vivo rat erythrocytes were investigated. For in vitro study, human carbonic anhydrase-I and -II (HCA-I and HCA-II) were purified by affinity-column chromatography, and rats were used for in vivo study. In vitro and in vivo CA enzyme activity was determined colorimetrically using the CO$_2$–hydration method of Wilbur and Anderson as modified by Rickli et al. Gentamicin sulfate (1.98—9.90 mM) showed in vitro inhibitory effects on HCA-I and HCA-II hydratase activity up to a 2 mM concentration, when determined using the CO$_2$–hydration method. Rat erythrocyte CA activity was significantly inhibited for up to 3 h ($p<0.001$) following intramuscular administration of gentamicin sulfate to Sprague-Dawley rats (3.2 mg/kg body weight). In conclusion, gentamicin sulfate inhibits CA enzyme activity in vivo and at low concentrations (=4 mM) in vitro.

Key words gentamicin sulfate; carbonic anhydrase; human; rat; erythrocyte

Carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) is present in nearly all organisms and catalyzes reversible hydration of CO$_2$ to HCO$_3^-$ and H$^+$. Seven distinct CA isozymes have been characterized from amniotes (primarily mammals). The only known physiological function of the CA isozymes is to facilitate the interconversion of CO$_2$ and HCO$_3^-$, and they therefore play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis.

Isozyme II (CA-II) is the most widely distributed CA in the eye, kidney, central nervous system (CNS), and inner ear. CA plays an important role in water and ion transport and pH regulation in the eye, CNS, inner ear, and some other systems. CA also plays a role in endolymph and cerebrospinal fluid synthesis and pH regulation due to the beneficial effects of acetazolamide on endolymphatic hydrops or hydrocephalus.

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 metabolic diseases, such as diabetes mellitus and hypertension. The inhibition of CA has been shown to impair H$^+$ secretion into the proximal small intestinal lumen, thereby decreasing bicarbonate resorption. In addition, the inhibition of CA leads to decreased acidification of urine, the production of alkaline urine, and eventually metabolic acidosis.

The CO$_2$–hydratase method of Wilbur and Anderson has been used to demonstrate the inhibition and activation effects of three different medical drugs on purified human CA (HCA) isozymes in our laboratory. It was observed that sodium ampicillin inhibited, and sodium dipyrone activated, while magnesium sulfate showed no effect on HCA-I hydratase activity. In addition, sodium ampicillin and magnesium sulfate inhibited while sodium dipyrone activated HCA-II hydratase activity. These observations have been demonstrated by in vivo studies in Sprague-Dawley rats in which erythrocyte CA activity was shown to be significantly inhibited by these drugs after 3 h.

In the present study, the effects of gentamicin sulfate, which had previously been shown to exhibit the greatest antibacterial spectrum among the aminoglycosides, were investigated on CA enzyme activity in in vitro human and in vivo rat erythrocytes.

MATERIALS AND METHODS

Materials Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from E. Merck (Merck KgaA, Darmstadt, Germany). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck. Gentamicin sulfate was provided by the University Hospital Pharmacy (Atatürk University, Erzurum, Turkey).

Purification of Human Erythrocyte CA Isozymes (HCA-I and HCA-II) by Affinity-Column Chromatography Erythrocytes were purified from fresh human blood (10 ml), which was obtained from the University Hospital Blood Center. Following low-speed centrifugation (3000×g for 15 min) (MSE, MISTRAL 2000) and removal of plasma anduffy coat, the red cells were isolated, washed twice with 0.9% w/v NaCl, and hemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were then removed by high-speed centrifugation (48745×g for 30 min) (Heraeus Sepatech, Suprafuge 22) at 4 °C and the pH of the hemolysate adjusted to 8.7 with solid Tris. The pH-adjusted hemolysate was then subjected to affinity-column chromatography column, 1.36×30 cm (Sigma Chemical); bed volume, 25 ml; peristaltic pump (Pharmacia, Uppsala, Sweden) and fraction collector (AO Instrument Company, U.S.A.) at 4 °C for the purification of HCA isozymes.

One hundred milliliters of pH-adjusted human erythrocyte hemolysate was applied to the Sepharose 4B-L-tyrosine-sulf-
fanylamide affinity column preequilibrated with 25 mM Tris–HCl/0.1 mM Na₂SO₄ (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na₂SO₄ (pH 8.7). The HCA isoforms (HCA-I and HCA-II) were eluted with 1.0 mM NaCl/25 mM NaH₂PO₄ (pH 6.3) and 0.1 M CH₃COONa/0.5 M NaClO₄ (pH 5.6), respectively (flow rate: 20 ml h⁻¹, fraction volume: 4 ml).

During the HCA isoform purification procedures, the absorbance at 280 nm was used to monitor protein elution by affinity-column chromatography. CO₂–hydratase activities in the eluates were determined and the active fractions were collected.12,13)

In Vitro Inhibition Studies The effect of increasing concentrations of gentamicin sulfate (1.98, 3.96, 5.94, 7.92, 9.90 mM) on HCA isoform activity was determined colorimetrically using the CO₂–hydratase method of Wilbur and Anderson as modified by Rickli et al.10,13,14) CO₂–hydratase activity as an enzyme unit (EU) was calculated from the equation ($t_0 - t_c$) where $t_0$ and $t_c$ are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively. The mathematical relationship between gentamicin concentration and CA activity (expressed as a percentage of that in the absence of gentamicin sulfate) was determined using conventional polynomial regression software (Microsoft Office 97, Excel).

In Vivo Inhibition Studies Ten adult Sprague-Dawley rats (200—250 g) were selected for intramuscular administration of gentamicin sulfate (3.2 mg · kg⁻¹ body weight). Blood samples (0.5 ml) were taken after each rat prior to gentamicin sulfate administration and at 1-, 3-, and 6-h intervals thereafter. They were placed in test tubes containing EDTA (2 mM) and subjected to centrifugation at 2500×g for 15 min at 4 °C (HERMLE Z383K). The erythrocyte pellets were 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 hemolysate. CA activity was determined colorimetrically as described above.12,13) Data are expressed as the mean ± S.D. Statistical analysis comprised significance testing of the difference between means (control vs. test) using the two-tailed Student’s t-test at the levels of 0.05, 0.01, and 0.001.

Protein Determination Quantitative protein determination was done by absorbance measurement at 595 nm according to the method of Bradford, with bovine serum albumin as the standard.15)

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) SDS-PAGE was performed after the purification of HCA-I and HCA-II isozymes. It was carried out at 10% and 4% acrylamide concentrations for the running gel and stacking gel, respectively, containing 0.1% SDS according to the method of Laemmli.16) The electrophoretic pattern was photographed (see Fig. 1).

Results and Discussion

When administered at relatively low doses, many chemicals affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme.17,18) The effects can be dramatic and systemic.19) Although gentamicin sulfate is used as a therapeutic antibiotic, its impact on CA activity has not previously been reported. Given the importance of CA in pH regulation in most tissues, the effects of increasing concentrations of gentamicin sulfate on human erythrocyte HCA-I and HCA-II isozymes was undertaken in this study. HCA-I and HCA-II were purified by Sepharose 4B-α-tyrosine-sulfanylamide affinity chromatography (Table 1) and the purity was confirmed by SDS-PAGE (Fig. 1). HCA-I and HCA-II isozymes were obtained with a yield of 39.5 and 22.4, and a specific activity of 10366, 52200 U/mg protein, and these enzymes were purified 1010- and 5087-fold, respectively (Table 1).

The range of gentamicin sulfate concentrations used was considered adequate to show enzyme inhibition or activation effects. It was evident from in vitro studies that the HCA-I and HCA-II were inhibited at gentamicin concentrations up to 2 mM, and activated at ≥4 mM (Figs. 2a, b).

Our data showed that there is correlation between the in vivo and in vitro effects of gentamicin sulfate on CA activity. If gentamicin sulfate 160 mg (molecular weight 477 g/mol) is administered intramuscularly in humans (weighing 60 kg, with approximately 5 L blood volume), a gentamicin sulfate concentration of approximately 0.067 mM is achieved. This concentration is within the range (up to 2 mM) which is inhibitory against in vitro CA-I activity (Fig. 2a).

After intramuscular injection, the serum gentamicin level reaches the peak concentration within 30—90 min. The half-

![Fig. 1. SDS-PAGE of HCA-I and HCA-II Purified by Affinity Gel](image)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (U/ml)</th>
<th>Total volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
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<tr>
<td>Hemolysate</td>
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<td>Sepharose-4B-α-tyrosine-sulfanylamide</td>
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<td>15</td>
<td>0.71</td>
<td>10.65</td>
<td>110400</td>
<td>10366</td>
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<td>Affinity-column chromatography</td>
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<td>0.1</td>
<td>1.2</td>
<td>62640</td>
<td>52200</td>
<td>22.4</td>
</tr>
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</table>

Table 1. Purification Scheme of HCA-I and HCA-II from Human Erythrocytes
zyme activity. These results show that there is a parallel life of gentamicin in plasma is 2—3 h. In our study, maximal inhibition of CA enzyme activity was seen within 1 h after drug administration and significant inhibition continued after 3 h (Table 2). These results show that there is a parallel between the peak plasma level of gentamicin and maximal inhibition of CA enzyme activity, and between the plasma half-life of gentamicin and continued of inhibition of CA enzyme activity.

It is generally recognized that CA controls the bulk of CO2 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 numerous secretory activities including fluid movements. 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 be diminished in the streptozotocin-induced diabetic rat. Gluconeogenesis and ureagenesis were also associated with an increase in hepatic CA-V activity. In addition, hepatic pH disequilibrium was explained in terms of changes in CA activity. Many drug side effects may 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 respiratory distress. Gentamicin has serious side effects in the kidney and/or inner ear. Owing to its ototoxic effects, gentamicin is used to decrease the amount of endolymph in Meniere’s disease therapy. This effect of gentamicin in the inner ear, at least in part, may be due to its inhibitory effect on CA-II enzyme activity. Moreover, development of metabolic acidosis during gentamicin therapy or impairment of excretion of an acid overload by gentamicin in metabolic acidosis may be related to inhibition of CA-II by gentamicin metabolite(s) in the kidney or in other tissue.

In conclusion, gentamicin sulfate showed in vitro and in vivo inhibition effects on erythrocyte CA activity. However, concentrations of gentamicin sulfate of ≥4 mM excessively augmented in vitro HCA-I and HCA-II activities. This inhibitory effect of gentamicin sulfate on CA activity may be one cause of its side effects. Additionally, increased side effects due to high dose of gentamicin sulfate administration are possible. The in vivo inhibitory effect on erythrocyte CA activity and associated sequelae should be considered in the therapeutic use of gentamicin sulfate. Administration of gentamicin sulfate to patients with metabolic acidosis can cause serious side effects and be deleterious to health. For this reason, gentamicin sulfate must be used carefully and the dosage should be closely monitored to decrease side effects.

REFERENCES

19) Botrè F., Botrè C., “Physiologic Implications of Carbonic Anhydrase

Table 2. Effect of Gentamicin Sulfate (3.2 mg · kg⁻¹ i.p.) on Rat Carbonic Anhydrase Activity

<table>
<thead>
<tr>
<th>Time after administration (h)</th>
<th>Carbonic anhydrase activity (EU/g Hb)⁶,⁷</th>
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<tr>
<td>0</td>
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<tr>
<td>1</td>
<td>17619.6±6080.0*</td>
</tr>
<tr>
<td>3</td>
<td>36816.9±2319.0*</td>
</tr>
<tr>
<td>6</td>
<td>53830.0±6205.0</td>
</tr>
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</table>

a) Results are expressed as the mean±S.D., n=10. b) Differences between means (test vs. control) were analyzed by Student’s t-test. *p<0.001. EU, enzyme unit.


