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Effects of Glycation on Erythrocyte Carbonic Anhydrase-I and II in Patients With Diabetes Mellitus

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Abstract: In order to investigate the effect of high blood glucose levels of long duration on carbonic anhydrase, and to evaluate the relation of the enzyme to its inhibitors in uncontrolled Type 1 diabetic patients, erythrocyte CA -I and CA-II activities and their kinetic parameters were determined. The effects of glycation on erythrocyte carbonic anhydrase-I and II (CA-I and CA-II) in patients with Type-I diabetes mellitus (uncontrolled) were investigated using kinetic parameters. After blood glucose and hemolysate total esterase activity levels had been measured in both 10 control and 10 diabetic subjects, CA-I and CA-II in hemolysate were purified separately by affinity chromatography. The enzyme activity, fructosamines, V_{max} , K_M, and K_i values of CA-I and CA-II were determined. The means of the blood glucose and hemolysate total esterase activity levels were significantly higher in the diabetics than in the controls ($p < 0.001$). After purification, the means of the enzyme activity, fructosamines, and V_{max} values of both CA-I and CA-II were significantly higher in the diabetics than in the controls ($p < 0.01$ $p < 0.001$ and $p < 0.01$) respectively, while the K_M values exhibited no significant differences ($p > 0.05$). The means of the K_i values of both CA-I and CA-II, using acetazolamide and sulfanilamide inhibitors, were significantly lower in the diabetics than in the controls ($p< 0.001$). Glycation was found to increase both CA activity and the inhibitory effect of acetazolamide and sulfonamide on CA activity. Since CA is a well-known enzyme regulating pH in most of the tissues in the body, changes in CA activity may be associated with metabolic diseases, especially in diabetes mellitus. Therefore, dosages of CA inhibitors should be considered carefully in the treatment of diabetic patients.

Key Words: Carbonic anhydrase, glycation, enzyme kinetics.

Introduction

Carbonic anhydrase (CA) is a well-characterized enzyme in all living organisms. CA is a pH-regulating enzyme in most tissues (1). Changes in CA activity have been associated with altered metabolism, especially in diabetes mellitus. Since glycation may cause changes in protein structure or enzyme function, leading to pathophysiologic complications of the disease, some studies on glycated-CA activity have been performed in diabetic patients.

Total CA (I+II+III+V) activity in liver was found to be diminished in streptozotocin-induced diabetic rat (2,3). It was also demonstrated that gluconeogenesis and ureagenesis increased with an increase in isozyme CA-V activity in hepatocytes and that the pH disequilibrium in rat liver could be explained by changes in CA activity (2,4). In addition, some studies on CA have been performed with erythrocytes from diabetic patients (5,6,7). The levels of both CA-I and CA-II were found to increase significantly in diabetic patients, while the activity levels showed no significant correlations (5). In another study, two different levels of CA activity were obtained in hypertensive patients (6). Recently, levels of total CA activity were found to increase significantly in patients with diabetes mellitus, hypertension and chronic renal failure, when compared with the control group (7).

Parenteral sulfonamides (i.e., acetazolamide and methazolamide) have been used for 40 years in therapy (8). In some studies, acetazolamide has been found to induce a dramatic fall in the glomerular filtration rate and cerebral blood flow in both diabetics and control subjects (9,10). It was concluded that these results could be associated with inhibition of CA in diabetics, since

sulfonamides are highly potent, selective, and reversible inhibitors of CA.

Since there has been no detailed report investigating the effects of glycation on CA isozymes in erythrocytes with diabetes mellitus, the aim of this study was to determine V_{max}, K_M and K_i values, using acetazolamide and sulfonamide on CA-I and CA-II purified from the erythrocytes of patients with diabetes mellitus. By using these kinetics parameters, adequate inhibitor dosages may be calculated. Thus, undesirable side-effects of these inhibitors on CA activity and body metabolism can be controlled.

Materials and Methods

The following chemicals were used in the study: Sepharose-4B, N,N,N',N'-tetramethyl ethylendiamine, L-tyrosine, and p-nitrophenylacetate were obtained from Sigma Chem. Co.; cyanogen bromide, p-aminobenzenesulfonamide, acetazolamide, acrylamide, Coomassie Blue G-250, and N,N'-methylene bisacrylamide from E. Merck; and Glyco-Probe GSP from Isolab. Inc.

Subjects: The blood samples were taken from uncontrolled diabetic patients. Ten uncontrolled diabetic patients (type I; 5 men and 5 women, 29 - 44 yr of age) and 10 controls (6 men and 4 women, 25 - 39 yr of age) were selected for the study. Venous blood samples with and without an anticoagulant were collected from all subjects after 12 h fasting. Fasting blood samples were obtained from the subjects and collected in tubes with and without heparin.

Blood Glucose Levels: Fasting blood glucose was assayed with the glucose oxidase method (11).

Total Esterase Activity In Hemolysate: The blood samples were centrifuged at 1500 rpm for 20 min. and the plasma and buff coat were removed. After the packed red cells were washed with NaCl (0.9 %), the erythrocytes were hemolyzed with chilled water. The ghost and intact cells were removed by centrifugation at 4 °C, 20,000 rpm for 30 min. The pH of the hemolysate was brought to 8.5 with solid Tris.

The total esterase activity was measured using the method described by Verpoorte et al. (12) with a slight modification. The assay system consisted of 100 µL of hemolysate containing 1.4 mL of 0.05 M Tris-SO_4 buffer (pH 7.4) and 1.5 mL of 3mM p-nitrophenyl acetate. The change in absorbance at 348 nm was measured over a period of 3 min., before and after adding hemolysate. One unit of enzyme activity was expressed as 1 µmol of released p-nitrophenol per minute at room temperature.

Purification of CA-I and CA-II Isozymes: The affinity chromatography column was prepared by using Sepharose 4B as a matrix, L-tyrosine as a spacer-arm and p-aminobenzene sulfonamide (sulfanilamide) as a ligand. The hemolysates obtained from all subjects were applied to the Sepharose-4B-L-tyrosine-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/ 0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with 25 mM Tris-HCl/ 22 mM Na_2SO_4 (pH 8.5). The CA-I and CA-II isozymes were eluted with solutions of 1 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 (13). The absorbance at 280 nm was used to monitor protein in the column effluents. The purified protein levels were determined by using the method for Coomassie Brilliant Blue with bovine serum albumin as a standard (14). The esterase activities of the purified CA-I and CA-II were measured by using 200 µL enzyme and 1.5 mM p-nitrophenyl acetate as described above. The purification was controlled by SDS-polyacrylamide gel electrophoresis (15).

Determination of Glycation of CA-I and CA-II: A nonkinetic colorometric method (Glyco-Probe-GSP) was used to determine the glycation levels of purified CA-I and CA-II from all the subjects (16). The standard solutions of 2.22, 3.25, and 4.04 mM were used for calculation. The assay for glycated CA is based on the ability of glucose to bind to the CA with a ketoamine linkage, i.e., to reduce a tetrazolium dye under alkaline conditions, and the resulting colour is measured at 500 nm.

Kinetic Studies: In all kinetic studies, 200 µL of the purified CA-I and CA-II were used. For obtaining the V_{max}, K_M and K_i values of CA-I and CA-II in the media without inhibitor and with inhibitor, the substrate concentrations were as follows: 0.6, 0.8, 1.0, and 1.2 mM of p-nitrophenyl acetate. For inhibition studies, 0.153, 0.306 and 0.460 mM of acetazolamide and 0.193, 0.387 and 0.581 mM of sulfanilamide were used as inhibitors. V_{max} and K_M values were obtained from Lineweaver-Burk graphs using esterase activities measured at four different substrate concentrations. K_i values were calculated from Lineweaver-Burk graphs using esterase activities measured at three different inhibitor concentrations for each substrate concentration.

Statistical Method: Values were expressed as the mean ± SD. The significance of the mean differences between the diabetic and control groups was assessed by Student's *t* test.

Results

The blood glucose concentrations and hemolysate total esterase activities of the diabetic and control groups are shown in Table 1 as X±SD. As expected, statistically significant differences were found between the diabetic and control groups in terms of the concentration of blood glucose and the enzyme unit of hemolysate total esterase. The means of blood glucose and hemolysate total esterase activity levels were significantly higher in the diabetic group (293.0±8.23 mg / dL and 5.11 ±0.23 U / gHb) than in the control group (106.4±2.11 mg / dL and 3.75±0.26 U / gHb) ($p < 0.001$). Both CA-I and CA-II in the diabetic and control subjects were purified from hemolysate, using an affinity column, for use in kinetics studies, and the purification was controlled with SDS-PAGE. As shown in Figure 1, in addition to the purification, the CA bands of the diabetic and control subjects were observed at the same molecular weight.

Some biochemical characteristics of CA-I and CA-II in the diabetic and control subjects after purification with an affinity column, are shown in Table 2. Significant differences were found between the diabetic and control groups in terms of the enzyme unit of esterase activity and the concentration of fructosamines. The mean esterase activity levels of both CA-I and CA-II, were significantly higher in the diabetic group (0.292±0.03 U / min / mL and 0.319±0.01 U / min / mL) than in the control group (0.267±0.06 U / min / mL and 0.290±0.03 U / min / mL) ($p < 0.01$). The means fructosamine levels of both CA-I and CA-II were significantly higher in the diabetic group ($2.91x10^{-2}$± $0.34x10^{-2}$ mmol/mg protein and $3.50x10^{-2}$±$0.25x10^{-2}$ mmol/mg protein) than in the control group ($1.03x10^{-2}$ ± $0.21x10^{-2}$ mmol/mg protein and 1.07 x 10^{-2}±$0.22x10^{-2}$ mmol/mg protein) ($p < 0.001$).

Finally, we examined the effects of the inhibitors on glycated CA. Kinetic parameters without and with inhibitors of purified CA-I and CA-II in the diabetic and control subjects are shown in Table 3. Significant differences were found between the diabetic and control groups in terms of the enzyme unit of V_{max} and the concentration of K_i with the exception of K_M. The means of V_{max} values of both CA-I and CA-II, were significantly higher in the diabetic group (1.97±0.08 U / min / mg protein and 4.03±0.11 U / min / mg protein) than in the control group (1.09±0.04 U / min / mg protein and 2.49±0.16 U / min / mg protein) ($p < 0.01$) (Table 3 and Figure 2). The means of K_i values of acetazolamide on both CA-I and CA-II were significantly lower in the diabetic group (0.38±0.02 mM and 0.23±0.02 mM)

Table 1. Clinical and biochemical characteristics of control and diabetic subjects.

	Subjects (n = 10)	
	Control	Diabetic
Blood Glucose (mg / dL)	106.4±2.11	293.0±8.23[a]
Hemolysate Total Esterase Activity (U / gHb)	3.75±0.26	5.11 ±0.23[a]

Values are expressed as mean ± SD.
[a]$p < 0.001$.

Table 2. Some biochemical characteristics of CA-I and CA-II in control and diabetic subjects purified with an affinity column.

	CA-I Subjects (n = 10)		CA-II Subjects (n = 10)	
	Control	Diabetic	Control	Diabetic
Esterase Activity (U / min / mL)	0.267±0.006	0.292±0.03[b]	0.290±0.03	0.319±0.01[b]
Fructosamine (mmol/mg protein)	$1.03x10^{-2}$±$0.21x10^{-2}$	$2.91x10^{-2}$±$0.34x10^{-2}$[a]	$1.07x10^{-2}$±$0.22x10^{-2}$	$3.50x10^{-2}$±$0.25x10^{-2}$[a]

Values are expressed as mean ± SD. [a]$p < 0.001$, [b]$p < 0.01$.

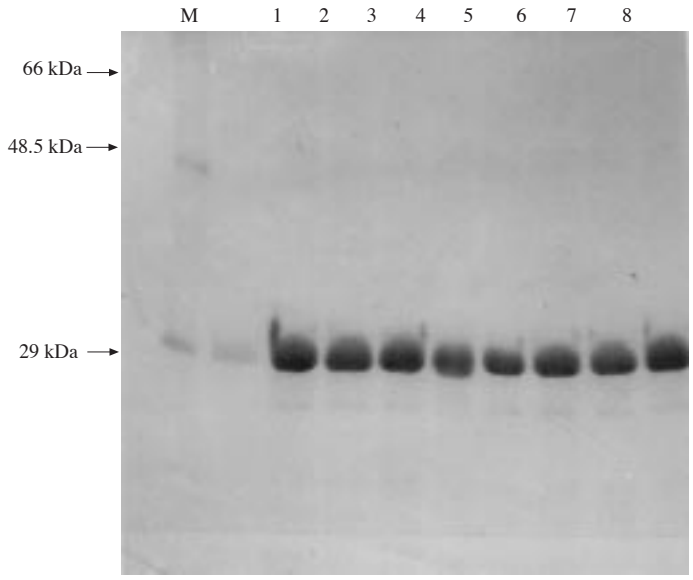

Figure 1. Electrophoretic pattern of erythrocyte CA-I and CA-II in two control and diabetic subjects purified with an affinity column. (M: Markers), (Lane 1 and 2: Control CA-I), (Lane 3 and 4: Diabetic CA-I), (Lane 5 and 6: Control CA-II) and (Lane 7 and 8: Diabetic CA-II).

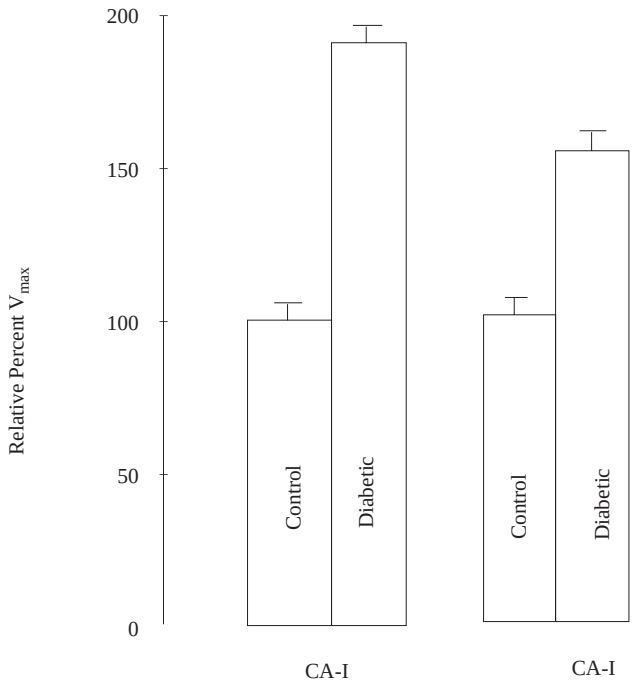

Figure 2. Percentage differences of V_{max} values of erythrocyte CA isozymes ($p < 0.01$).

than in the control group (0.63±0.07 mM and 0.40±0.01 mM) ($p < 0.001$) (Table 3 and Figure 3). The means of K_i values of sulfanilamide on both CA-I and CA-II were significantly lower in the diabetic group (0.50±0.02 mM and 0.31±0.02 mM) than in the control group (0.70±0.03 mM and 0.54±0.04 mM) ($p < 0.001$) (Table 3 and Figure 3). In these diabetic subjects, the K_M values of both CA-I and CA-II were similar to those of the control group ($p > 0.05$).

Table 3. Kinetic parameters without and with inhibitors of CA-I and CA-II in control and diabetic subjects purified with an affinity column.

	CA-I Subjects (n = 10)		CA-II Subjects (n = 10)	
	Control	Diabetic	Control	Diabetic
Vmax (U / min / mg protein)	1.09±0.04	1.97±0.08[b]	2.49±0.16	4.03±0.11[b]
K_M (mM)	2.16±0.16	2.29±0.20[c]	3.71±0.29	3.20±0.62[c]
K_i , (mM) (ACZ)	0.63±0.07	0.38±0.02[a]	0.40±0.001	0.23±0.02[a]
K_i , (mM) (SLF)	0.70±0.03	0.50±0.02[a]	0.54±0.04	0.31±0.02[a]

ACZ = Acetazolamide, SLF = Sulfanilamide. Values are expressed as mean ± SD.
[a]$p < 0.001$, [b]$p < 0.01$, [c]$p > 0.05$.

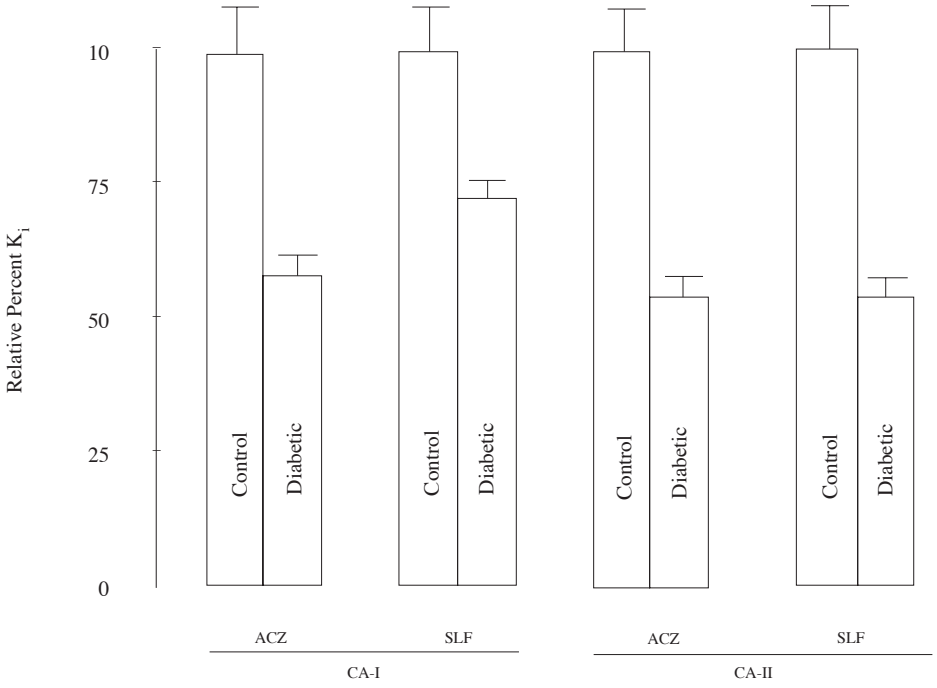

Figure 3. Percentage differences of K_i values of erythrocyte CA isozymes ($p < 0.001$). ACZ = acetazolamide, SLF = sulfanilamide.

Discussion

Since nonenzymatic glycation may cause changes in protein structure or enzyme function leading to pathophysiologic complications of the disease, nonenzymatic glycation has been studied in hemoglobin (17), plasma albumin (18), lens crystallins (19), plasma apolipoproteins (20), erythrocyte membrane proteins (21), and erythrocyte carbonic anhydrase (CA) (5,6,7).

Especially in diabetes mellitus and hypertension (7), changes in CA activities have been associated with altered metabolism, because CA is a pH-regulatory enzyme in most of the tissues (1). There have not been any studies examining the effects of glycation on CA-I and CA-II separately purified from erythrocytes with diabetes mellitus on the basis of kinetic parameters. Therefore, in this study, we investigated V_{max}, K_M and K_i values by using some inhibitors on CA-I and CA-II.

First, blood glucose and hemolysate total esterase activity levels were determined (Table 1). Both levels were higher in the diabetic group than in the control group ($p < 0.001$) as in previous studies (3,5,6,7). After this observation, CA-I and CA-II were purified from the hemolysates obtained from all the diabetic and control subjects. As shown in Figure 1, the CA bands in the diabetic and control subjects were observed at the same molecular weight. The esterase activity levels of each purified isozyme were found (Table 2). The esterase activity levels of both CA-I and CA-II were higher in the diabetic group than in the control group ($p < 0.01$), as Kondo *et al.* also observed (5).

Fructosamine levels were measured to determine the glycation of the purified CA-I and CA-II (Table 2). The fructosamine levels were higher in the diabetic group than in the control group for both CA-I and CA-II ($p < 0.001$). In order to show the effects of glycation on CA isozymes, in this study, for the first time, kinetics parameters were determined using acetazolamide and sulfanilamide, since acetazolamide has been shown to induce a dramatic fall in glomerular filtration rate and cerebral blood flow in both diabetic and control subjects (9,10). Hannedoeche et al. and Rodriguez et al. concluded that these results can be associated with the inhibition of CA in diabetics since sulfonamides are a highly potent, selective, and reversible inhibitor of CA. Therefore, in the present study, this inhibition of CA was investigated by determination of K_i values on CA-I and CA-II purified from erythrocytes with diabetes mellitus. As given in Table 3, the means of the V_{max} values of both CA-I and CA-II were significantly greater in the diabetics than in the controls ($p < 0.01$), while the K_M values showed no significant correlations ($p > 0.05$). Since nonenzymatic glycation may cause changes in protein structure or enzyme function , the increased V_{max} values and unchanged K_M values suggest that glycation on CA increases catalytic activity, since it does not occur in an active site. As shown in Table 3, the means of the K_i values on both CA-I and CA-II, determined using acetazolamide and sulfanilamide inhibitors were significantly lower in the diabetic group than in the control group ($p < 0.001$). The lower K_i values (40 % on CA-I and 42% on CA-II for acetazolamide, 29 % on CA-I and 43 % on CA-II for sulfanilamide), as shown in Figure 3, may mean that glycation on CA increases the affinity of CA to these inhibitors.

Carbonic anhydrase is well known as a pH-regulating enzyme in most tissues, including erythrocytes (6). It has been reported that the activity levels of CA isozymes in human erythrocytes vary considerably under certain pathological and physiological conditions. Changes in CA activity have been associated with metabolic diseases like diabetes mellitus and hypertension (6,7). Since acetazolamide has been found to induce a dramatic fall in glomerular filtration rate and cerebral blood flow in both diabetic and control subjects, it has been concluded that these results can be associated with the inhibition of CA in diabetics (9,10). These results also indirectly suggest that there is an altered basal tubulo-glomerular feedback system and altered cerebrovascular reactivity. When acetazolamide is used to investigate the altered systems, the acetazolamide dosage should be considered according to K_i values on glycated CA isozymes obtained in the present study.

It has been reported that the inhibition of CA was found to impair proton secretion into the proximal tubular lumen, thereby decreasing bicarbonate reabsorption. At the same time, the inhibition of CA also decreased the rate of the acidification of urine, producing alkaline urine and eventually metabolic acidosis (9). When parenteral sulfonamides (i.e., acetazolamide and methazolamide, carbonic anhydrase inhibitors) are used in therapies, considering these kinetic parameters obtained in the present study, useful inhibitor dosages may be calculated. Thus, undesirable side-effects on CA activity and body metabolism can be reduced in the therapy of diabetes mellitus. In addition to this, the effects of nonenzymatically glycated forms of other erythrocyte enzymes should be studied so as to understand the pathological aspects of red cell metabolism in diabetes mellitus.

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