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In vitro inhibition of human erythrocyte hexokinase by various hyperglycemic drugs

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Abstract

Hemolysis is the red blood cell abnormality most often associated with adverse effect of drug therapy. Drug-induced or drug-associated hyperglycemia could decrease the activity of hexokinase. The aim of this study was to investigate the inhibitory effects of some commonly used drugs that have hyperglycemic side effect on the human erythrocyte hexokinase enzyme *in vitro*. Hexokinase was purified from human erythrocytes using sequential chromatography, with a specific activity of 0.96 ± 0.18 U/g hemoglobin, and assayed in the presence of selected drugs that have hyperglycemic side effect. The IC_{50} were determined from the regression analysis graph. Correlation analysis showed that there was positive correlation between the hyperglycemic side effect of some of the tested drugs and decrease of hexokinase activity. This suggests that, at least in part, these drugs exert their hyperglycemic effect by inhibiting glucose phosphorylation by the hexokinase, which consequently causes the glucose accumulation.

KEYWORDS

erythrocyte, glucose metabolism, hemolysis, hexokinase, hyperglycemia

1 | INTRODUCTION

The ATP-dependent phosphorylation of glucose is the first reaction in glycolysis, and is catalyzed by tissue-specific isoenzymes known as hexokinases I, II, III, and IV.[1] Red blood cells hexokinase subtype (HK-R) is encoded by the same gene as Hexokinase I (HK-I) and is mainly present in erythroblasts, reticulocytes, and young erythrocytes.[2] HK-I is more stable with a longer half-life and replaces HK-R as the erythrocyte matures[3] and, as a result, mature erythrocytes contain only 2%–3% of the HK-R activity of reticulocytes.[4]

In the process of maturation, the respiratory capacity of the cell is lost and the metabolic activity greatly reduced. Thus, the energy requirement of the mature erythrocyte is derived largely through the anaerobic glycolysis, and, to a lesser extent, through the oxidation of glucose by way of the pentose phosphate metabolic pathway.

Hexokinase deficiency is a very rare glycolytic enzymopathy associated with nonspherocytic hemolytic anemia NSHA.[5,6] Hexokinase deficiency results in hemolysis of variable degree, from severe neonatal hemolysis requiring exchange transfusion to a fully compensated chronic hemolytic anemia.

Drugs and chemicals that inhibit hexokinase enzyme and cause decrease in erythrocyte energy may reflect cumulative increased oxidative random destruction.[7] Drug-induced hyperglycemia occurs due to a variety of drugs and mechanisms.[8–11] Mechanisms range from decreased insulin secretion to decreased insulin action to direct neurological effects.[12] There are several additional hyperglycemic medications that appear to have still undefined mechanism.

Clinicians need to be aware not only of the list of drugs that may lead to hyperglycemia but also understand the underlying pathogenic mechanisms. Knowledge of potential mechanisms by which a medication can disrupt glucose metabolism may help guide optimal therapy for medication-induced premature erythrocytes destruction in their patients. One scenario among the possible mechanisms of drug-induced hyperglycemia may include hexokinase inhibition.

Hydrochlorothiazide, furosemide, pantoprazole, propranolol, chlorpromazine, diltiazem, atorvastatin, clozapine, gabapentin, valproic acid, gatifloxicin, indomethacin, and enalaprilat had remarkable hyperglycemic side effects and could decrease the activities of hexokinase. These drug effects may be dramatic in patients suffering from erythrocyte hexokinase deficiency. Therefore, in the present study, investigation of the effects on the activity of human erythrocyte hexokinase of some commonly used drugs that have hyperglycemic side effect was proposed.

2 | MATERIALS AND METHODS

Sephacryl S-300, FPLC equipment, and MonoQ were obtained from Amersham Buckinghamshire, UK. Other chemicals and biochemicals used in this work were of analytical grade and purchased from Merck Darmstadt, Germany Sigma–St. Louis, MO USA.

2.1 | Enzyme preparation

Blood sample obtained from a healthy volunteer was freed of leukocytes and platelets by passing through a cellulose column,[13] and the erythrocytes were washed three times with isotonic saline and packed by centrifugation. The packed erythrocytes were lysed with 1 vol of 0.4% saponin in H_2O for 1 h. After centrifugation at 30,000*g* for 30 min, the supernatant was loaded on DEAE-cellulose (DE52) equilibrated with (10 mM KH_2PO_4, 20 mM Tris–Cl, pH 7.4, 0.5 mM dithiothreitol, and 0.1 mM EDTA). After washing with equilibration buffer, the enzyme was eluted with 500 mM KH_2PO4 in the same buffer and precipitated with 60% saturated ammonium sulfate. After standing overnight at 4°C, the precipitate was collected by centrifugation, was dissolved in equilibration buffer, and was dialyzed against the same buffer. The protein fraction was loaded on the MonoQ column. Following washing, the adsorbed proteins were eluted with a 20–300 mM KCl gradient in the same buffer at 0.5 mL/min. Fractions containing hexokinase activity were pooled and applied to a Sephacryl S-300 column, subsequently eluted with equilibration buffer containing 50 mM KCl. The fractions containing hexokinase activity were pooled, concentrated by ultrafiltration, and stored at −20°C. Before use, the stored enzyme was dialyzed against 10 mM Tris–HCl, pH 8.0. During the purification procedure, protein was determined according to the method of Lowry et al.,[14] with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

2.2 | Kinetics studies on human erythrocyte HK

Hexokinase activity was assayed spectrophotometrically, through a coupled reaction with glucose-6-phosphate dehydrogenase, following the $NADP^+$ reduction at 340 nm at 37°C for 10–20 min.[15] The assay mixture contained: 100 mM Tris–HCl pH 8.5 mM $MgCl_2$, 0.8 mM ATP, 1 mM $NADP^+$, 3 international units of glucose-6-phosphate dehydrogenase, and glucose concentrations at the concentrations indicated in the text. The inhibitory effects of the 13 drugs on the hexokinase activity of human erythrocytes were investigated. Hydrochlorothiazide, furosemide, indomethacin, atorvastatin, clozapine, chlorpromazine, and valproic acid were dissolved in DMSO. Propranolol, diltiazem, enalaprilat, gatifloxacin, pantoprazole, and gabapentin were dissolved in water. Different drugs concentrations (0–100 μM) were added to the incubation mixture described above to investigate their inhibitory effects. The enzyme activity in the absence of drug was used as a control (100% activity). The enzyme was incubated with each drug for 10 min before the assay.

2.3 | Data analysis

All data are shown as mean ± SEM of at least three independent experiments (each performed in triplicate). The inhibitor concentrations causing up to 50% inhibition IC_{50}s and maximal effect were determined by fitting the data to a one-site competition model with nonlinear regression using GraphPad Prism 7. Means were compared by ANOVA with Dunnett's multiple comparison posttest. The Dixon plot data were analyzed by fitting the data with linear regression.[16] In order to determine Ki constants for the inhibitors, the substrate [Glc] concentrations were 1 and 10 mM. Inhibitors (drugs) solutions were added to the reaction medium, at six different fixed concentrations of inhibitors in 1 mL of total reaction volume.

TABLE 1 The Half Maximal Inhibitory Concentration (IC_{50}) and Maximal Inhibition of Tested Drugs

Drug	IC_{50} (μM)	% Maximal inhibition
Hydrochlorothiazide		0
Pantoprazole		0
Propranolol		0
Furosemide		0
Atorvastatin	44	45
Gabapentin	30	54
Enalaprilat	10	75
Gatifloxacin	8	73
Clozapine	6	68
Indomethacin	5	85
Valproic acid	34	65
Chlorpromazine	77	50
Diltiazem	33	51

All compounds were tested on four independent enzyme preparations.

3 | RESULTS

The mechanisms of drug-induced hyperglycemia up to now are uncertain, but several factors have been postulated to be involved,[17] it is a complex and probably multifactorial process. Putative mechanisms may include hexokinase inhibition.

We evaluated this hypothesis and the possible correlation between the hyperglycemic side effect of some drugs and decrease of hexokinase activity by assessing the human erythrocytes hexokinase activity in the presence of selected drugs that have hyperglycemic side effect.

Hexokinase was purified from human erythrocytes using sequential chromatography, with a specific activity of 0.96 ± 0.18 U/g hemoglobin, and assayed in the presence of hydrochlorothiazide, furosemide, pantoprazole, propranolol, chlorpromazine, diltiazem, atorvastatin, clozapine, gabapentin, valproic acid, gatifloxicin, indomethacin, and enalaprilat all at concentrations as low as 100 μM (which are therapeutically attainable)

Addition of some of these drugs to the reaction mixture resulted in a marked inhibition of the hexokinase activity. Figure 1 shows the inhibition of erythrocytes hexokinase enzyme by selected hyperglycemic agents. Indomethacin, clozapine, enalaprilat, and atorvastatin significantly inhibited human erythrocyte hexokinase and furosemide did not inhibit hexokinase activity. Table 1 shows the inhibitor concentrations causing up to 50% inhibition (IC_{50}) values and the maximal amount of inhibition by all the compounds tested. IC_{50} values of atorvastatin, gabapentin, enalaprilat, indomethacin, clozapine, gatifloxacin, valproic acid, chlorpromazine, and diltiazem were 44, 30, 10, 5, 6, 8, 34, 77, and 33 μM, respectively.

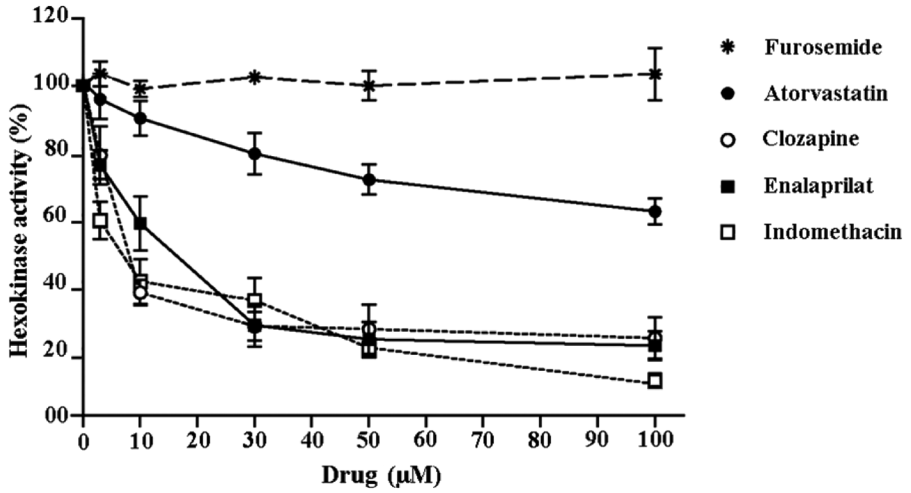

FIGURE 1 Inhibition of erythrocytes hexokinase enzyme by selected hyperglycemic agents. The effects of furosemide, atorvastatin clozapine, indomethacin, and enalaprilat on erythrocytes hexokinase enzyme activity are shown as indicated. Hexokinase activity in the absence of inhibitor served as the control. Where not shown, error bars are smaller than the symbols

IC_{50} values show that the enzyme is mostly inhibited by clozapine, indomethacin, gatifloxacin, and enalaprilat drugs. Hydrochlorothiazide, furosemide, pantoprazole, and propranolol had no effect. Addition of atorvastatin, gabapentin, valproic acid chlorpromazine, and diltiazem to the reaction mixture resulted in a marked inhibition of the hexokinase activity only at high doses.

The inhibitor concentrations that show significant, though not complete, inhibition are in the low μM range. The half maximal inhibitory concentration (IC_{50}) of the most potent compounds is around 10 μM.

Analysis of indomethacin and atorvastatin inhibition by Dixon plot suggests a noncompetitive inhibition (Figure 2). The atorvastatin inhibition was not as marked as it was with indomethacin. The inhibitory constants Ki for indomethacin and atorvastatin were 15 ± 1 and 23 ± 3 μM, respectively. Since higher affinity drugs have lower Ki values, these results show that indomethacin has a twofold higher affinity for the hexokinase than atorvastatin, whilst the affinity of the other compounds are approximately sixfold less than indomethacin.

IC_{50} values show that indomethacin had the highest inhibitory effect, followed by clozapine, gatifloxacin, and enalaprilat. Ki constants exhibited almost similar inhibitory patterns.

4 | DISCUSSION

Hexokinase plays a key role in the metabolic regulation of the red blood cell because the energy production in red blood cells is derived entirely through glycolysis, and hexokinase is considered to be an important factor in erythrocyte senescence .[18] In aged red blood cell, many enzymatic activities, including glycolytic enzymes such as hexokinase, sharply declines during the ageing processes which could lead to an increase in the rate of endogenous reactive oxygen species (ROS) generation, and oxidative stress can cause hemolytic episode when some drugs are used in enzyme-deficient patients.[19–22]

In general, 25% percent of the patients with residual HK-I activity suffer from chronic nonspherocytic hemolytic anemia (CNSHA) to a variable degree.[23,24] The continuous lack of sufficient energy and enhanced accumulation of non-phosphorylated glucose resulting from the HK-I deficiency may favor (ROS) generation and hemoglobin glycation within red blood cells, which in turn may increase the RBC death rate via their impaired deformability.[25] The survival of erythrocytes in the microcirculation system depend primary on their deformability. A decrease in cell deformability may result from a direct or indirect oxidative damage of the cell membrane. Knowledge of potential mechanisms by which a medication can disrupt glucose metabolism may allow us to avoid many of these reactions.

As in the case of hexokinase enzyme deficiency, exposure to certain drugs and chemicals that inhibit hexokinase enzyme and cause decrease in erythrocyte energy may results in increased premature erythrocytes random destruction. This was directly demonstrated to be the case by monitoring the inhibitory effects of the 13 drugs on the hexokinase activity of human erythrocytes *in vitro*, the hexokinase activity was markedly lowered when exposed to some hyperglycemic agents (Table 1).

If the abovementioned offending drugs inhibited hexokinase activity, this would lead to the induction of oxidative stress and to an increased level of intracellular glucose, which, if sufficient, would decrease the rate of glucose influx and, hence, cause a decrease in the net rate of glucose transport.

Based on the findings from the above studies, we postulated that HK-I inhibition may modulate the maintenance of glucose 6-phosphate pool and thus indirectly alter the blood glucose concentration.

Hexokinase activity of the erythrocytes treated with atorvastatin, gabapentin, valproic acid, gatifloxacin, indomethacin clozapine, and enalaprilat was decreased to 15%–30% of normal (Table 1), which may have important clinical implications for the enzyme-deficient patients. From these initial screening studies, using the obtained Ki constants and IC_{50} as cut-off values, undesirable side effects of these drugs on hexokinase activity and erythrocytes deformability can be reduced.

It was seen that hydrochlorothiazide, furosemide, pantoprazole, and propranolol have no significant effects on hexokinase activities *in*

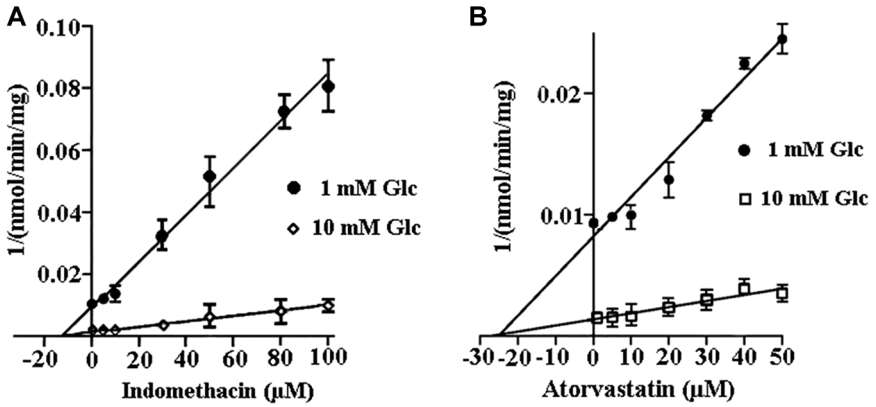

FIGURE 2 Dixon plots of indomethacin and atorvastatin inhibition of erythrocytes hexokinase enzyme. The data are plotted as the inverse of the velocity versus the concentration of inhibitor (Indomethacin A or Atorvastatin B) and represent the means of three experiments. The *lines* show the results of linear regression of the data. The inhibitory constants were determined by the *x*-intercept

vitro. Thus, these drugs can be used in therapy in the presence of hexokinase deficiency. Additionally, atorvastatin, gabapentin, valproic acid chlorpromazine, gatifloxacin, diltiazem, clozapine, indomethacin, and enalaprilat should not be given to the hexokinase-deficient patient. If it is required to give these drugs to the patient with hexokinase deficiency, dosage should be strictly determined to decrease the hemolytic side effect. While there is a general lack of data and recommendations for treatment of medication-induced hyperglycemia, close monitoring of hemolysis is recommended where the abovementioned drugs are used in patients suffering from hexokinase deficiency. Therapy includes stopping the drug use and transfusions when anemia is severe.

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