Original Article

Aminoguanidine partially prevents the reduction in liver pyruvate kinase activity in diabetic rats

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Abstract

Background: Low molecular weight aldehydes and carbonyl compounds which are derived from glucose metabolism are prevalent in diabetic plasma. These compounds react to amino groups of Lys and Arg and lead to the formation of advanced glycation end products (AGEs). This modification changes the function of the proteins. The present study aimed to survey the effect of diabetes on rat liver pyruvate kinase activity and to show the inhibitory effect of aminoguanidine (AG).

Materials and Methods: Male Wistar rats ($n = 18$, 6 to 8 weeks old) were divided randomly in three groups: the first group as control; second and third groups were induced diabetes using streptozocin. Third group received AG orally for 8 weeks after diabetes induction. Liver cell homogenate was prepared from all studied groups and L-type pyruvate kinase was separated from the homogenate. Pyruvate kinase activity was determined in both liver cell homogenate and extracted L-type PK. The PK activity was compared in all samples between groups.

Results: PK activity in isolated form and in liver cell homogenate was lower in diabetic rats as compared to control group. AG-treated group showed higher PK activity compared to untreated diabetic group; however, the difference was not significant. Non-significant difference in PK activity between AG-treated diabetic and non-diabetic (control) group indicated the inhibitory effect of AG in glycation of PK.

Conclusion: The obtained results showed PK activity decreased in diabetic rats and AG can partially prevent the reduction in PK activity.

Key Words: Aminoguanidine, diabetes, liver, pyruvate kinase, rat

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INTRODUCTION

Diabetic plasma contains a large amount of glucose and also low molecular weight aldehydes and carbonyl compounds which are derived from glucose metabolism or related physiological substrates.[1,2] These compounds include glyoxal and 3-deoxyglucosone (3-DG) which are derived from glucose;[3,4] methylglyoxal (MG) a derivative of glucose, glyceraldehyde-3-phosphate, acetone, aminoacetone,[5,6] and glycolaldehyde which are derived

from glucose and amino acid oxidation.[4-7] Reaction of these compounds to amino acid side chains, especially to amino groups of Lys and Arg and lead to the formation of advanced glycation end products (AGEs).[8] There is a relation between AGEs and diabetes and many aging pathologies such as atherosclerosis, cataract, neuropathy, and Alzheimer.[2-9]

Aminoguanidine (AG) containing two parts, i.e. a nucleophilic hydrazine and a guanidine group scavenges dicarbonyl compounds.[10] It strongly blocks protein glycation and also prevents the formation of AGEs *in vivo* and *in vitro*.[11]

There are evidences showing that AG has an effective role in inhibition of AGEs formation and prevention of diabetic complications.[12,13]

Pyruvate kinase (PK, ATP-pyruvate-O-phospho transferase, EC 2.7.1.40), which plays a metabolic role in bacteria, plants, and vertebrates, is found in various organisms. In mammalian tissues, there are 4 types of PK which are as follow: M_1 isoenzyme that is found in skeletal muscle, heart and brain; M_2 isoenzyme that is present in kidneys, adipose tissue, and lungs; R isoenzyme that exists in erythrocytes, and L isoenzyme found in liver.[14-16]

PK isoenzymes control consumption of metabolic carbon for biosynthesis and use of pyruvate for energy production.[17] The regulatory role of L-PK plays a crucial role in living organisms.[18,19] Researches on glycation of intracellular proteins show high susceptibility of the enzymes to modification with MG.[20-22]

Despite many research studies on the importance of glycolysis in production of MG as a glycation factor, little data are available about modification of glycolitic enzymes. It has been shown that MG inhibits glycolysis in metastatic cells of Ehrlich tumor and leukemic leukocytes through the formation of glycated glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[23,24] Furthermore, glycation of GAPDH and LDH from rabbit muscle as a result of treatment with MG leads to a significant decrease in these enzyme activities.[25,26]

A recent study in PK activity on cultured fibroblasts isolated from type 1 diabetic patients with and without nephropathy showed a significant decrease in PK activity in diabetic fibroblasts with nephropathy compared to those of subjects without nephropathy and normal people.[27]

The present study aimed to survey the effect of diabetes on PK activity from diabetic rat liver both in cell homogenate (M_2 and L isoenzyme) and L-PK isoenzyme extracted by DEAE-Cellulose column chromatography. The second aim was to show the effect of AG on prevention of PK glycation.

MATERIALS AND METHODS

Aminoguanidine (AG), DEAE-Cellulose, Streptozotocin (Streptozocin, STZ), N-acetylcysteine, 1-Naphthyl phosphate monosodium salt monohydrate, Fast Red RC, Tris base, and electrophoresis marker were purchased from Sigma-Aldrich (USA). Monoclonal mouse anti rat L-PK was from ScheBo•Biotech AG (Giessen, Germany). Pyruvate Kinase Activity Assay Kit was prepared from Biovision (California, USA) and Bovine anti-mouse IgG from Santa Cruz (Santa Cruz, U.S.A.).

Animal treatment
The experimental protocols were approved by the Ethical Committee of Hamadan University of Medical Sciences (Iran). Male Wistar rats, 8 weeks old (body weight, 150-180 g) were purchased from Pasture Institute (Iran).

First, to acclimatize the rats to their new environment, they were maintained in the new environment for 1 week before the start of the study. All rats were housed in a room with controlled environment, at a temperature of ~25°C, normal humidity, and a 12-hour light/dark cycle. The animals had access to standard rat chow and water *ad libitum* throughout the experimental period. After that, the rats were randomly divided into three groups: diabetic (D), controls (C), and diabetic treated with aminoguanidine (DA) of 6 animals. Body weight of each animal was monitored weekly during the period of the study.

Induction of diabetes
In groups D and DA, diabetes was induced via intraperitoneal injection with a single dose of STZ (65 mg/kg), diluted in citric acid buffer (0.1M, pH 4.5). Control group was injected with the same volume of citric acid buffer.

Blood glucose was measured before the administration of STZ, after 72 hours and at the end of 2nd, 4th, 6th, and 8th weeks by taking blood samples from the tail vein using a glucometer (GLUCOCARD 01 ARKRAY Company, Japan).

Diabetes was confirmed by the presence of hyperglycemia through tail vein sampling 72 hours after STZ administration. STZ-treated animals having blood glucose more than 250 mg/dl were considered diabetic.

Treatment with aminoguanidine

After confirmation of diabetes, group DA was offered a solution of 0.1% (w/v) aminoguanidine *ad libitum* for 8 weeks.

Tissue preparation

At the end of 8th week, all animals were anesthetized using chloroform vapor and were sacrificed. The livers were excised, washed in ice-cold saline to remove their blood, frozen using liquid nitrogen, and immediately transferred to -80° C for storage until use.

The livers were homogenized using a Heidolph DIA × 900 Homogenizer at 0- to 4°C, with 1 volume (w/v) of a solution of 0.5 M sucrose, 25 mM Tris HCl, 2.5 mM EDTA, 4 mM acetylcysteine, 2.5 mM $MgCl_2$ at pH 7.5. The homogenates were centrifuged for 1 hour at 40,000 g in a Sigma 3K 30 at 0 to 4°C. After that, the supernatant solutions were carefully removed and filtered through glass wool to remove their fat pad.

Protein assay

Protein in the cell lysate supernatants and eluted fraction from column were measured using the Bradford method, with bovine serum albumin as a standard.[28] All assays were performed in triplicate.

Enzyme activity assay

Pyruvate kinase activity in cell lysate supernatants and extracted L-type were measured by Pyruvate Kinase Activity Assay Kit (BioVision). In the assay, reaction of PEP and ADP was catalyzed by PK to generate pyruvate and ATP. The generated pyruvate was oxidized by pyruvate oxidase to produce color product and the absorbance was measured by a spectrophotometer (λ = 570 nm). All assays were performed in triplicate. Unit definition for pyruvate kinase activity: one unit of pyruvate kinase was the activity of enzyme that transfers a phosphate group from PEP to ADP yielding 1.0 μmol of pyruvate per minute at 25°C.

Separation of L-type by DEAE-cellulose

All separation procedures were performed in cold room at 0-4°C. The clear and fat-free supernatant solutions were transferred directly to a column, 600 × 10 mm, containing a depth of 400 mm of DEAE-cellulose, prepared according to the method of Peterson and Sober.[29] The column was equilibrated with a pH 7.5 solution containing 0.25 M sucrose, 10 mM Tris HCl, 1 mM EDTA, 1mM $MgCl_2$, and 4 mM acetylcysteine. Then, the column was washed with 100 ml of the same solution, and elution was conducted with a linear KCl gradient with 500 ml of this solution in chamber 1 and 500 ml of the same buffer solution containing 0.5 M KCl in chamber 2.[30] With the use of a peristaltic pump, the flow rate was adjusted to 25 ml/hr, and 5 ml fractions were collected.

To compare the activity of isolated L-type pyruvate kinase between different groups, the fraction that had maximum activity from each group was selected and PK-specific activity was determined.

Electrophoresis and immunoblotting

SDS-PAGE on isolated samples was performed using Laemmli method.[31] To confirm the presence of L-type, the separated proteins in electrophoresis gel were transferred to a nitrocellulose membrane (Sigma) using electroblotting, and the transferred bands were visualized using related antibody and alkaline phosphatase-conjugated secondary antibody.

Statistical analysis

Mann-Whitney U-test was used for comparing the results. $P < 0.05$ was considered statistically significant.

RESULTS

Body weights and plasma glucose levels

Table 1 shows the mean ± SD body weights of animals in each group at the beginning and at the end of study. The body weights of D and DA groups changed slightly whereas the body weights of groups C increased significantly ($P < 0.05$). The mean plasma glucose concentrations of diabetes rats increased significantly compared to those of control group ($P < 0.05$).

Enzyme activity and specific activity of the liver cell homogenates are shown in Table 2. As this table shows, PK activity and specific activity in the group D decreased compared to the control group ($P < 0.05$). However, there was slight increase in PK activity and specific activity in the group DA compared to the group D, the difference was not significant.

L-type pyruvate kinase was isolated using DEAE-ion exchange chromatography and the purification was checked using SDS-PAGE and western blotting (data not shown).

As Table 3 shows, L-type PK activity and specific activity in group D decreased compared to the control group ($P < 0.05$), while L-type PK activity and specific activity showed slight but non-significant increase in group DA compared to group D.

PK activity in both cell homogenate and isolated enzyme in the treated animals with AG did not show significant difference as compared to those of control (non-treated group), indicating the prevention of reduction in PK activity.

Table 1: Mean±SD of body weights at start and end of study, and plasma glucose levels in different rat groups before and after diabetes induction

	Body weights (g)		Plasma glucose (mg/dl)	
Groups (*n*=6)	Start	End	Start	After 72h Inj
C	157±7.04	315±16.58*	107±40.0	-
D	154±6.01	148±11.45	98±14.11	451±95.3*
DA	162±8.05	179±12.29	102±20.49	512±69.5*

*Significant difference (D vs C) and C: Control rats, D: Diabetic rats, DA: Diabetic rats treated with aminoguanidine

Table 2: Activity and specific activity of pyruvate kinase in the rat liver cells homogenate in different studied groups

Groups (*n*=6)	Activity (U/ml)	*P*	Specific activity (units/mg)	*P*
C	17.947±1.149	-	0.177±0.01	-
D	16.172±1.108	0.045*	0.158±0.012	0.045*
DA	17.468±1.051	0.128**	0.162±0.014	0.688**

*Significant difference (D vs C) and ** non-significant difference (DA vs C), C: Control rats, D: Diabetic rats, DA: Diabetic rats treated with aminoguanidine

Table 3: Activity and specific activity of L-type pyruvate kinase extracted from liver cell homogenates in different rat groups using DEAE-cellulose chromatography

Groups (*n*=6)	Activity (U/5ml)	*P*	Specific activity (units/mg)	*P*
C	8.532±0.948	-	2.047±0.343	-
D	7 0.047±0.929	0.045*	1.318±0.428	0.02*
DA	8.180±1.077	0.092**	1.592±0.563	0.298**

*Significant difference (D vs C) and ** non-significant difference (DA vs C), C: Control rats, D: Diabetic rats, DA: Diabetic rats treated with aminoguanidine

DISCUSSION

Hyperglycemia is one of the clinical manifestations of diabetes which causes several complications.[32] It was shown that in diabetic rats compared to healthy animals, some plasma factors such as glucose and fructosamine increased that lead to glycation of proteins.[33] Also, there is some evidence showing reactive oxygen species (ROS) increase in people suffering from hyperglycemia which is due to protein glycation.[34]

Furthermore, it is shown that in patients with diabetes mellitus, plasma levels of free radicals increased compared to healthy people because of protein glycation.[35] Therefore, increased glucose concentration is an important factor in developing diabetes-associated complications.

Our results showed a decrease in PK activity and PK specific activity in diabetic group (in liver cell homogenate and extracted L-type) compared to control group ($P < 0.05$).

Although in agreement with the previous reports, our results confirmed that diabetes causes decrease

in PK activity, the mechanisms of inhibition are not clear completely, this may in part be due to the lack of insulin or post-translation modifications of the enzyme or both of them. Therefore, more studies are needed to explain the exact mechanisms. Also, research studies especially on the post-translation modifications of PK in diabetes are scarce.

Comparing the specific activity of pyruvate kinas between the AG-treated diabetic group (DA) and diabetic group (D) showed the higher specific activity in DA group; however, the difference was not significant. Furthermore, compared to the control group, the specific activity did not change significantly, indicating the AG effect. This effect can be due to prevention of glycation by AG; however, the other mechanisms cannot be ruled out.

In a study, it was shown that insulin caused a significant increase in the activity of liver PK in diabetic rats.[36] This increase in PK activity was due to a dual mechanism, including a significant increase in the amount of the enzyme and a mild increase in its specific activity.[36]

Investigation of the effect of AG on inhibition of PK glycation and prevention of PK activity reduction is very complicated because the PK activity is changed through different ways. In diabetic rats treated with AG, PK activity can be affected by inhibition of post-translation modification by AG and lack of insulin. Therefore, in the present study, AG may in part prevent post-translation modification of enzyme and also prevent the reduction of enzyme activity; however, this reduction can be due to lack of insulin.

Research shows many controversies about the activity and mRNA expression of PK in diabetes. The present study is in accordance with a number of research studies. It has been shown that PK activity and its mRNA expression are decreased both in adipose tissue and cultured pancreatic islets of T1DM patients.[37,38] In another research in which insulin-dependent animal models were used, it was pointed out that there is a decrease in activity and mRNA expression of PK.[39] However, in contrast to these reports, some researchers have shown that PK activity either increases or remains unchanged in diabetes.[40,41]

AG is α,β-dicarbonyl scavenger which prevents protein glycation and therefore it inhibits formation of AGEs. There is a relation between AGEs and age-related disorders and complications in diabetes.[10,42]

In a study, it was shown that hydrazine part of AG reacts with other carbonyl groups such as pyridoxal,

some quinone compounds (enzyme cofactor), and harmful free aldehydes.[43] In addition, this research reported that AG not only reacts to formaldehyde and methylglyoxal *in vitro* quickly but also it can scavenge the aldehydes *in vivo*.[43]

Another research showed that lipid hydroperoxide levels are higher in diabetic compared to healthy rats, which indicates that chronic hyperglycemia can cause cellular damage. This damage can probably happen through the oxidant compounds attack to membrane lipids.[33]

While it has been shown that AG cause a significant decrease of lipid hydroperoxide level, low lipid hydroperoxides levels in rats treated with AG may be due its antioxidant activity.[33]

It was confirmed that glycation of the antioxidant enzymes could not be discharged in rats treated with AG because the amounts of fructosamine reduced significantly.[33] This result indicates that protein glycation is an important factor of the damage caused by hyperglycemia to the antioxidant system. The cellular oxidative damage induced by free radical generation and therefore the capacity of the cell to inhibition the attack of free radicals may be reduced by the glycation.[33]

In a study, increase in catalase gene expression was observed in diabetic rats but enzyme activity decreased in them. This data indicated a role for post-translation modification in changing the activity of this enzyme with glycation.[44]

In another study, effect of glycation in antioxidant defenses in rats treated with AG has been discussed. In this research, the high levels of HbA_{1c} and fructosamine in diabetic rats indicate that the glycation process is active; however, the treatment with aminoguanidine reduced the levels of fructosamine (35%). Thus, this finding indicated that AG partly inhibited glycation process.[33,45]

In conclusion, the current findings indicated a significant decrease in PK activity and PK-specific activity in diabetic group (in cell homogenate and in isolated isoenzyme) that may in part be due to post-translation modification of this enzyme or lack of insulin. In addition, slight (however non-significant) increase in L-PK activity and specific activity in the DA group in comparison with group D and also no significant difference in the activity of PK between DA group and C groups (control) support this hypothesis that AG prevents reduction in the PK activity in diabetic rats. These findings indicate that AG may partly prevent post-translation modification of PK.

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