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In Vitro Study of Glycation of Pyruvate Kinase and Its Effect on Activity and Proteolytic Resistance In Absence And Presence Of Aminoguanidine

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ABSTRACT

Protein glycation participates in structural changes that impair protein functionality and this process is related to pathogenesis of diabetes and several diseases. This in vitro study was aimed to examine the effect of glycation on pyruvate kinase (PK) activity and proteolytic resistance, also to show the inhibitory activity of aminogaunidine (AG) in these processes.

PK from rabbit muscle was treated with 4 mM MG or 3-DG and also in presence of 12 mM AG at 37 º C in the dark for 30 days in PBS buffer (pH 7.5). Activity of PK was determined using a spectrophotometric kit. PK was treated with trypsin and changes in electrophoretic pattern was studied using SDS-PAGE.

The results showed a non significant decrease in the activity of PK treated with MG compared to un-treated PK. Treatment in presence of AG also showed a non-significant difference in PK activity compared to the control. Similar results were observed with 3-DG. Electrophoresis of treated PK with MG or 3-DG in presence and absence of AG did not show any differences in the patterns. Furthermore trypsin treatment of modified PK did not show different proteolysis products.

In conclusion we can indicate that PK was resistant to glycation in the reaction condition used in this study. Also it is possible that PK molecular weight alteration by glycation was not in a level detectable by SDS-PAGE. *In vivo* studies of PK in diabetic patients or animal models using mass specrtrometry can reveal more details.

KEYWORDS: glycation , pyruvate kinase , proteolytic resistence , aminoguanidine.

INTRODUCTION

Pyruvate kinase (PK , ATP-pyruvate-o-phosphotransferase , EC2.7.1.40) is found in various organisms such as bacteria , plants and vertebrates and play a key role in metabolism of these 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 and lungs tissues; R isoenzyme that exists in erythrocytes and L isoenzyme is found in liver [1, 2]. These isoenzymes control consumption of metabolic carbon for biosynthesis and use of pyruvate for energy production [3].

Glycation is a biologic process that results to formation of advanced glycation end-product (AGE) and it has effective role in the pathogenesis of diabetes and its complications [4]. AGE-proteins are produced by post-translational modification through glucose reaction with free amino groups of various molecules [5]. Metabolic disorders such as diabetes contains a large amount of glucose with low molecular weight aldehydes and carbonyl compounds which is derived from glucose metabolism or related physiological substrates [6].

Methylglyoxal (MG) is an α-oxoaldehyde compound, produced in all mammalian cells by enzymatic and non-enzymatic mechanisms. The large quantity of MG produces from the glycolytic intermediates, which includes fragmentation and elimination of phosphate from triose phosphate [7]. Furthermore, it is generated in the ketone body metabolism and in the catabolism of threonine. This molecule is very reactive, but its interactions with proteins are controlled by glyoxalase [8].

3-Deoxyglucosone (3-DG) is an aliphatic aldehyde with physiological significance which has been reported to participate in AGEs formation with proteins. This molecule produces through Maillard reaction and also from fructose-3-phosphate via the polyol pathway [5]. 3-DG reacts with protein amino residues and produces AGEs such as carboxymethyllysine (CML), 3-DG-imidazolone, pyrraline, and pentosidine [9]. 3-DG-imidazolone and pyrraline are markers of 3-DG derivative AGEs *in vivo*. It was reported that 3-DG-imidazolone is the most product in 3-DG derivative AGEs [10].

Recent researches on aminoguanidine (AG) and AGEs have been led to the strategy of using AG for breaking the existing AGEs cross-links [11]. In addition, more and more evidences show that this molecule has an effective role in prevention of diabetic complications [12]. Therefore it has important clinical implications in treatment of

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disorders which are related to AGEs. Nevertheless, the potential side effects of AG need serious attention [13]. However there is great hopes that the molecule will be used as a drug in the future [14].

Various researches that are carried out on the glycation of intercellular proteins show the high susceptibility of enzymes to modification with MG [15-17]. Despite many researches on the importance of glycolysis in production of MG as a glycation factor, limtted data is available about modification of glycolitic enzymes. It has been shown that MG inhibits glycolysis in metastatic cells of Ehrilich tumor and leukemic leukocytes through the formation of glycated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [18,19]. Furthermore, glycation of GAPDH and LDH from rabbit muscle as a result of treatment with MG leads to a significant decrease in these enzymes activities [20 , 21].

The present study designed to study the effect of MG and 3-DG on the activity and proteolytic resistence of pyruvate kinase. Also the effect of aminoguanidine on inhibition of the effects of MG and 3-DG on the activity and proteolytic resistence of pyruvate kinase was studied.

MATERIALS AND METHODS

Aminoguanidine (AG), 3-deoxyglucosone (3-DG), methylglyoxal (MG), trypsin from bovine pancreas, pyruvate kinase from rabbit muscle, L-Lysine hydrochloride and molecular weight marker were purchased from Sigma-Aldrichand (USA). Also pyruvate kinase activity assay kit was obtained from Biovision (California , USA) .

Treatment of enzyme with MG, 3-DG and AG:

Treatment of PK with MG, 3-DG and AG were carried out at 37º C in the dark for 30 days. The experiments were performed in PBS buffer pH 7.5 using 5 µM of PK (concentration 1.185 mg/mL, specific activity 538 U/mg) and an excess of methylglyoxal (4 mM approx. a 800 molar ratio of MG to enzyme) and also an excess of AG 12 mM. (approx. a 3 molar ratio of AG to MG) in appropriate samples. All of the similar protocols were repeated with 3-DG. In other word, all the above steps were performed but MG was substituted with 3-DG. The high molar levels of MG and 3-DG were used for maximal saturation of accessible reactive amino acid residues in the PK.

The glycation process was terminated by the addition of 18 mg/mL lysine solution to bind the uncoupled glycotoxines. After 30 days, aliquot of samples were taken to measure enzyme activity, SDS-PAGE analysis and proteolytic digestion.

Proteolytic digestion of samples by trypsin:

A vial of 100 µg of lyophilized bovine pancreas trypsin (320 U) was dissolved in 1 mL of 15 mM Tris-HCl buffer, pH 7.3, containing 10 mM $CaCl_2$. After 30 days from treatment of enzyme with MG, 3-DG and AG, the reaction was stopped by the addition of lysine solution. 100 µl samples containing 120 µg of PK were treated with 16 µl of protease solution at 25º C for 3h. Aliquot of samples were taken from the mixture for SDS-PAGE.

Enzyme Activity Assay:

Pyruvate kinase activity of all PK specimens were measured using pyruvate kinase activity assay Kit (BioVision, USA). In the assay, reaction of PEP and ADP were catalyzed by PK to generate pyruvate and ATP. The generated pyruvate was oxidized by pyruvate oxidase to produce color product and the absorbance was measures by a spectrophotometer (λ=570 nm) . All assays were performed in triplicate.

Electrophoresis:

SDS-PAGE was performed using Laemmli method [22]. The final acrylamide concentration in the slab gels was 10 % for the separating gel and 5% for the stacking gel. Before electrophoresis all samples were incubated for 5 min at 100° C in the presence of 50 mM Tris HCl (pH 6.8) containing 50 mM 2ME, 50% glycerol, 10% SDS and 1% bromophenol blue. The electrophoresis was performed at 100 V and 150 V for stacking and separating gels respectively (EPS-600Z , Tehran , Iran). Finally, the gel stained using Coomassie Brilliant Blue R-250.

Statistical analysis:

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

RESULTS

As shown in Table 1, a slight decrease in activity of PK treated with MG was observed, however the difference was not statistically significant. Also a non significant difference in activity of PK treated with MG and AG samples compared to their controls were observed ($P > 0.05$) . In addition, Table 2 indicates a non significant

reduction of activity in PK treated with 3-DG and also PK treated with 3-DG and AG groups compared to their controls ($P > 0.05$).

The SDS-PAGE pattern of effects of 4 mM MG and also 3-DG on modification of rabbit muscle PK and prevention effects of AG revealed the same pattern for all samples including modified and un-modified PK (Figure 1). Furthermore the SDS-PAGE pattern of susceptibility of glycation products derived from rabbit muscle PK to proteolytic degradation by trypsin was similar for all groups (Figure 2). Therefore, the results showed that in these protocols MG and 3-DG were not able to induce significant changes in the activity and SDS-PAGE pattern of PK. However AG could partly prevent decrease of PK activity comparing to their controls.

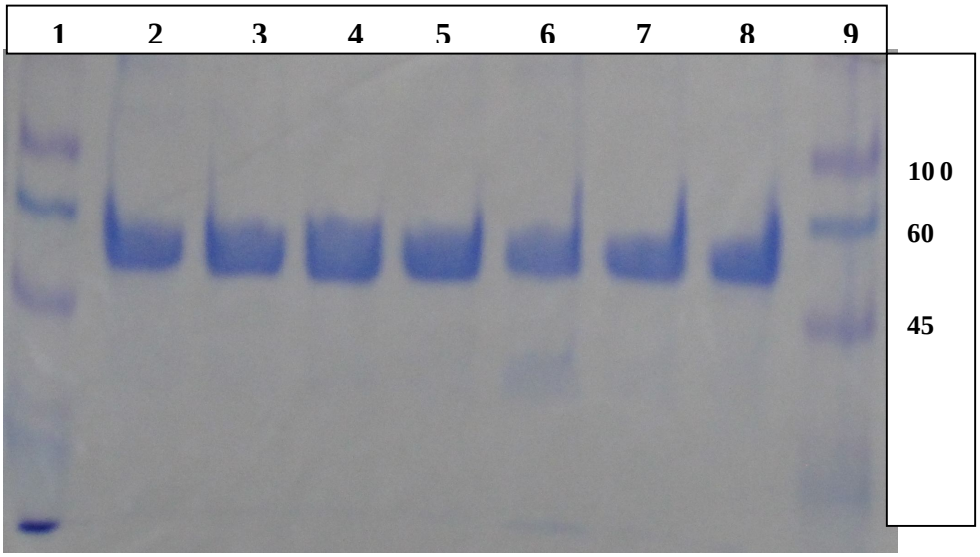

Figure 1. Electrophoretic pattern of rabbit muscle PK treated with 4 mM MG or 3-DG in presence and absence of AG.

Lanes 1and 9: Molecular weight marker, lane 2: PK + MG , lane 3: PK (control), lane 4: PK + MG+AG , lane 5: PK +AG, lane 6: PK + 3-DG , lane 7: PK + 3-DG + AG, lane 8: native PK.

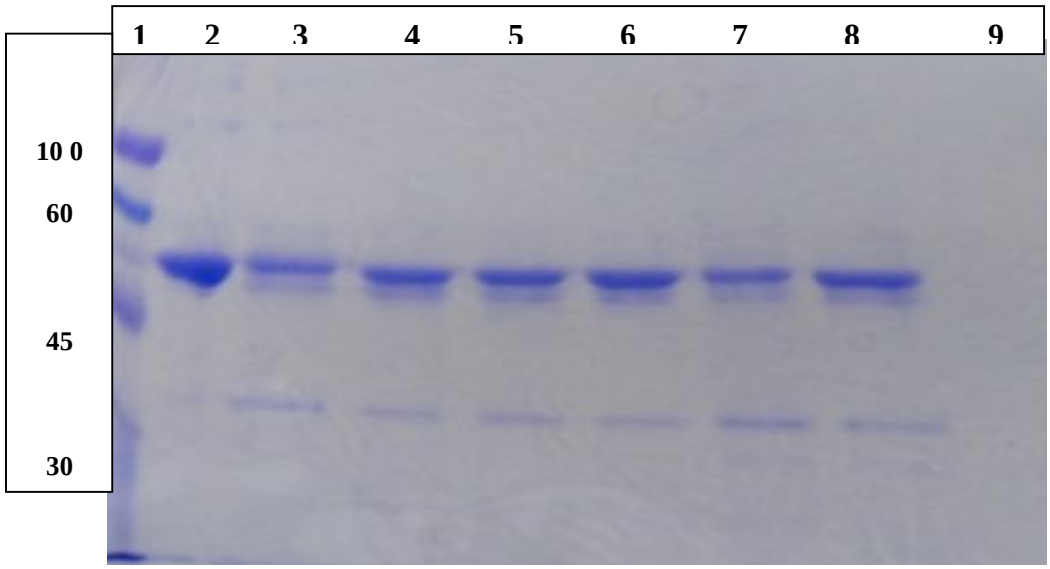

Figure 2. Electrophoresis pattern of proteolysis of native and glycated PK.

lane 1: Molecular weight marker, lane 2: PK , lane 3: PK + MG+ Trypsin, lane 4: PK + trypsin (control), lane 5: PK + MG + AG +trypsin, lane 6: PK + AG + Trypsin , lane 7: PK +3-DG + Trypsin ; lane 8 PK + 3-DG +AG + Trypsin, lane 9: Trypsin.

Table 1 . Mean of PK activity in PK treated with MG and PK treated with MG and AG comparing to their controls

Groups	Activity(U/ml)
PK (control)	618 ± 12.51
PK + MG	602 ± 11.63
PK + MG + AG	629 ± 10.81
PK + AG (control)	625 ± 14.08

No significant differences were observed between groups .

Table 2 . Mean of PK activity in PK treated with 3-DG and PK treated with MG and 3-DG with their controls

Groups	Activity(U/ml)
PK (control)	611 ± 11.26
PK + 3-DG	593 ± 14.18
PK+3-DG + AG	617 ± 11.45
PK + AG (control)	623 ± 13.51

No significant differences were observed between groups .

DISCUSSION

MG may modify various proteins, even at physiological concentrations. Under the physiological concentrations, most of the produced MG is bound to guanidine group of arginines, amino group of lysines and sulfhydryl group of cysteines [23, 24]. However its reaction with the SH-group of cysteine is reversible and their change is not a severe threat to cells. Increasing levels of MG may lead to irreversible changes of arginine and lysine residues and produce AGEs [24, 25].

3-DG is participated in aging and involves in renal disease and pathogenesis of diabetes. It is shown that its plasma concentration increase in uremic disease and in diabetic patients [26, 27]. Researches based on plasma assay of 3-DG and its urinary concentration suggested a relationship between defected detoxification of this carbonyl and nephropathy [28].

3-DG participation in modification of body proteins has been confirmed by immunohistochemistry and immunochemistry in diabetic and uremic diseases [29]. Another study showed 3-DG-imidazolone aggregate in nodular lesions matrix and renal arteries in diabetic nephropathy and in atherosclerotic lesions of the aorta. Also, pyrraline has been obsereved in sclerosed glomeruli, arteries with arteriosclerosis and plaques of patients with Alzheimer [30]. Additionally, higher pyrraline amount has been shown in cataractous lenses [31].

3-DG derivatives were accumulated in the extracellular matrix and in intracellular lesions. In addition, 3-DG-imidazolone and carboxymethyllysine may bind AGE receptor on macrophages and smooth muscle cells and induce production of cytokines and growth factors that are involved to the pathology of diabetic vascular disease [32].

Our results indicated that AG slightly prevented reduction of PK activity in presence of MG, however the difference was not statistically significant. Also similar results were observed when PK was treated with 3-DG in presence of AG. These finding can show the inhibitory activity of AG on carbonylation of PK.

The electrophoresis analysis of rabbit muscle PK after treatment with MG and 3-DG and also in presence of AG revealed the same pattern, indicating that carbonylation modification did not led to molecular weight changes that can be detected by SDS-PAGE.

Also, the electrophoresis pattern of proteolysis products on glycated and un-glycated rabbit muscle PK did not show any differences, indicating that glycation did not have any effect on proteolysis resistance. Therefore, these results showed that in these protocols MG and 3-DG could not significantly change the activity and

electropheretic mobility of PK. However AG could partly prevent decreasing of PK activity comparing to the controls.

In a study it was shown that MG reduced activity of human muscle-specific enolase [33]. The treated enzyme with MG was susceptible to a progressive decrease in activity. The amount of activity inhibition depended on the concentration of the MG and the kind of buffer. When incubation of enzyme was performed in PBS for 180 min with 2 mM, 3.1 mM and 4.34 mM MG, significant reductions in activity were observed (32%, 55% and 82% of the initial activity respectively). The amount of inactivation of this enzyme by MG relates to the concentration of inhibitor and the time of reaction [33]. Also, similar results are reported for GAPDH and LDH from rabbit muscle after treatment by MG [20, 21].

In addition, other researches showed the similar dependence related the activity of the other enzymes, such as glycolytic enzymes [16, 17, 20, 34]. The decrease of enzymatic activity is caused by the binding of inhibitor to the lysine and arginine present in the active site and other parts of the enzyme. The small size of MG enables this molecule to enter to the active site of enolase and inhibit the arginine and lysine that are important in the reversible change of 2-PGA to PEP [33]. Glycated enolase indicates an increase of unordered conformation *in vivo*. α-Helical content reduces and *T*m increases with modification, indicating that glycated enzyme may exist in a more compact and rigid structure [35].

When enolase was modified *in vitro*, besides an enhanced increase in unordered conformation and a reduction in α-helix, an increased gain of β-sheet was shown [35]. The *T*m increases and indicates an even more rigid conformation, may be due to higher β-sheet. In addition, unfolding is much more observed [35]. This may be due to the increase of glycation or due to the molecular chaperone pathway that is activated by modification *in vivo* but is absent *in vitro* [36, 37]. The observed alterations in protein conformation and stability are resulted to the glycation-dependent activity reduction, 65% inactivation *in vivo* and 85% activity decrease *in vitro* [35].

Other research indicated that MG may interact directly with lysine residues, leading to covalent crosslinking of Cu, Zn-SOD [38]. The results also showed that the inactivation of Cu, Zn-SOD may be closely associated with the loss of histidine residues, since this amino acid is essential for Cu, Zn-SOD activity [38].

In a research on crude enzyme extracts of glucokinase, hexokinase, glucose-6-phosphatase, G6PD and phosphofructokinase from mouse liver treated with 3-DG indicated that glucokinase and G6PD activity were decreased slightly but hexokinase activity was severely inhibited [39]. Also, it was demonstrated that glucose-6-phosphatase and phosphofructokinase activity were scarcely affected [39].

The most defects in the used protocol in this research were the important differences between *in vitro* and *in vivo* glycation processes. *In vitro*, non physiological amounts of glycotoxines are used, from millimolar to molar amounts [40-42, 16]. Also protein interactions, protein turnover and the action of chaperones, some of them are activated upon glycation by methylglyoxal is not present *in vitro* [36, 37,43]. These differences highlight the importance of investigating of protein glycation processes and their biochemical interactions *in vivo.* Additionally, another study using mass spectrosmetry analysis demonstrated that *in vivo* glycation of enolase is site-specific and only a few amino acid residues are consistently modified, and it has been concluded that protein glycation is different *in vivo* and *in vitro* [35]. Furthermore this *in vitro* glycation is a heterogeneous process, resulting in the production of a complex population of enolase molecules with different glycation structures. Also, different MAGEs (methylglyoxal derived AGEs) may bind at the same arginine residue, in different proteins *in vitro* [35].

Overall, our data indicated that glycation of PK resulted from treatment with MG or DG leaded to a slight but non-significant decrease on its enzyme activity. Also presence of AG in glycation reaction of PK slightly prevented the decrease in activity of PK. Furthermore we showed that the glycation of PK did not affect its susceptibility toward proteolysis. What can be concluded from these finding is that *in vitro* glycation of PK did not change its activity and proteolytic resistance. However it is important to point out that *in vitro* glycation in this study did not change the molecular weight of PK, indicating that PK was resistant to glycation in the reaction condition used in this study. The other possibility is that in this study PK molecular weight alteration by glycation was not in a level detectable by SDS-PAGE. Mass-specrpmetry studies are required to analyze exact changes in PK. Also *in vivo* studies of PK in diabetic patients or animal models can reveal more details.

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