Hypoglycemic Effects of D-glutamic Acid in Diabetic Rats

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Abstract: The present study was designed to investigate the hypoglycemic effects of D and L isomers of glutamic acid in diabetic rats through inhibiting phosphoenolpyruvate carboxykinase. The results of this in vitro study showed meaningful inhibitory effects by glutamic acid isomers to phosphoenolpyruvate carboxykinase activity. Groups of 10 rats were used as control and treated groups. Diabetes in experimental rats was induced by administering streptozotocin (STZ) (60 mg kg⁻¹, i.p., once). The effect of L and D glutamic acids on blood glucose was studied by injecting doses of 100 mg kg⁻¹ b.wt. intra peritoneally to groups of diabetic and control rats. Present results showed that the D isomer of glutamic acid causes a significant decrease in blood glucose 1.5 h post-injection with continued activity up to 4 h. The L isomer as previously reported resulted in hyperglycemia, probably because of its involvement as a precursor in the gluconeogenesis pathway. Following up the changes in serum protein, urea and triglyceride concentration we observed no significant alterations in their concentrations indicating no side effects for D-glutamic. We found evidences that D-glutamic acid may be considered for further pharmaceutical experiments as a hypoglycemic drug.

Key words: Phosphoenolpyruvate carboxykinase, inhibition, D-glutamic acid, diabetes mellitus, gluconeogenesis

INTRODUCTION

Phosphoenolpyruvate carboxykinase, EC number 4.1.4.32, is the first enzyme in gluconeogenesis (Croneiger et al., 2002); it catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (PEP) as shown in the following equation.

\[ \text{Oxaloacetate} + \text{GTP} \rightarrow \text{PEP-HCO}_3^- + \text{GDP} \]

Cellular compartmentalization of phosphoenolpyruvate carboxykinase is species dependent. It is cytosolic in mouse, rat and hamster (Croneiger et al., 2002; Hakimi et al., 2005) mitochondrial in chicken and pigeon and both cytosolic and mitochondrial in rabbit, pig and human (Yang et al., 2009; Giffin et al., 1993; Watford and Tatro, 1989; Cornell et al., 1986). Normally, phosphoenolpyruvate carboxykinase takes part in glucose production under fasting conditions (Yang et al., 2009; Quinn and Yeagley, 2005; Cadoulal et al., 2008). The cytosolic form of phosphoenolpyruvate carboxykinase is under hormonal control. In human, the hormonal control is exerted on the cytosolic enzyme in order to match the physiologic need, whereas, the mitochondrial enzyme is unaffected by hormones (Xu et al., 2005; Hanson and Reshef, 1997; Drewnowsk et al., 2002). There are reports that lack of cytosolic form of phosphoenolpyruvate carboxykinase causes prolonged hypoglycemia and even results in death (Hakimi et al., 2005; She et al., 2000, 2003; Hanson and Reshef, 1997; Beale et al., 2002). The increase or decrease of the enzyme biosynthesis is induced by absence or presence of insulin respectively (Rajas et al., 2000; Fränckhauser et al., 2002). In Insulin Dependent Diabetes Mellitus (IDDM), the increase of cytosolic form of phosphoenolpyruvate carboxykinase results in hyperglycemia (De Fronzo and Ferramini, 1991; Cadoulal et al., 2008). Infusion of amino acids, the building blocks of proteins, has different effects on gluconeogenesis. Amino acids such as Ala and Gin result in hyperglycemia (Dorf et al., 2007). Also, amino acids such as Leu desensitize target cells against insulin causing hyperglycemia (Anthony et al., 2000; Yoshizawa, 2004; Baum et al., 2005). Whereas, some other amino acids produce hypoglycemic effects by enhancing insulin production in beta cells of pancreas (Khamzina et al., 2005; Takano et al., 2001; Tappy et al., 1992, 1994) or by producing metabolites like quinolimic acid, as is the case with Trp, which induce hypoglycemic effects via inhibition of gluconeogenesis (Dayer et al., 2009). The fact that gluconeogenesis is the predominant glucose generating pathway in fasting conditions which

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is abnormally accelerated in diabetic conditions, makes it an alternative target for designing drugs with hypoglycemic effects. Conducting this in vitro kinetic study of phosphoenolpyruvate carboxykinase inhibition by oxaloacetate analogues we found that L and D enantiomers of glutamic acid have profound inhibitory effects on enzyme activity. Given the controversial reports about adverse effects of commercially used monosodium glutamate in food industry (Win, 2008) and the findings of our study, we decided to undertake the present study to investigate the inhibitory effects of L and D glutamic acids on phosphoenolpyruvate carboxykinase activity both in vitro and in vivo, in an attempt to find out whether the adverse effects are exerted via glucose metabolism or not.

MATERIALS AND METHODS

All chemicals were purchased from Sigma and Aldrich chemical companies, 2008. The experiments have been performed in biochemistry facilities of the Department of Biology of Shahid Chamran University (SCU).

Diabetes production: Diabetes was induced in rats by intraperitoneal injection of streptozotocin dissolved in 0.9% sodium chloride at a dose of 60 mg kg⁻¹ of b.wt. This relatively high dose of streptozotocin was suitable to obtain a sever model of diabetes mellitus with the lowest secretion of insulin from beta cell of pancreas so as to minimize the interference of insulin with inhibitors action. Diabetic rats with blood glucose values of over 300 mg/100 mL, measured by the glucose oxidase method, were used as a diabetic model a week after the streptozotocin injection. The experimental procedures were conducted with due attention to the guidelines for the care and use of laboratory animals approved by the Research Ethical Committee of Chamran University.

Injection of inhibitor: L and D glutamic acids at a dose of 100 mg kg⁻¹ b.wt. were dissolved in an appropriate volume of saline and were injected intraperitoneally to animals.

Enzyme purification: Phosphoenolpyruvate carboxykinase was purified from rat liver cytosol according to the method of Ballard and Hanson (1969) and Chang and Lane (1966) as follows: groups of ten wistar rats (ten rats in each group) weighting about 300 g were starved for 24 h prior to sacrificing. This treatment increased the amount of phosphoenolpyruvate carboxykinase activity. The animals were sacrificed 2 h post injection a blow to the head, their livers were quickly removed and placed in ice-cold 0.25 M sucrose for enzyme purification. Rat livers were homogenized in 2 volume of 0.25 M buffered sucrose using a coxial homogenizer and centrifuged at 100,000×g for 1 h to obtain 450 mL of clear supernatant. Sufficient solid ammonium sulfate was added to the supernatant from the previous step to bring the solution to 45% saturation. It was usually necessary to add some 1 M Tris during this fractionation to maintain the pH level of enzyme solution at 7.0. After 15 min the suspension was centrifuged at 5000×g for 15 min and the precipitate was discarded. Additional solid ammonium sulfate was added to increase the saturation percentage of the solution to 65% and after 15 min this suspension was centrifuged as previous step. The precipitate was dissolved in 0.05 M Tris-chlorid, pH 8 to a volume of approximately 75 mL. All purification steps were carried out at 0-4°C to avoid denaturation or instability of the enzyme at higher temperatures.

Enzyme assay: The assay used in all experiments was essentially as reported by Chang and Lane (1966) and modified by Ballard and Hanson (1969). The enzyme activity was determined in conversion of oxalacetate to phosphoenolpyruvate direction. To this end 240 units of Malate dehydrogenase, 2 mM MnCl₂, 0.0536 g malate and 0.0527 g NAD⁺ are dissolved in 200 mM Tris-HCl pH 7.5 and incubated in a water bath at 37°C for 1.5 h. After this period of time the absorbance at 340 nm was constantly zero. Then the buffer pH was brought to 8 by adding enough sodium hydroxide. Adding the purified enzyme, at this stage, triggered the enzymatic reaction, resulted in NADH accumulation and increased the absorbance at 340 nm. The enzyme activity was measured by plotting absorbance change at 340 nm against time and initial slope kinetics was considered. Enzyme assays were carried out using a Shimadzu model UV-3100 (Japan) spectrophotometer and a thermostatically controlled cell compartment with a Haak D8 water bath.

Determination of insulin and C-peptide: The tail vein of experimental rats was bled to obtain 1 mL of blood in order to quantify glucose, insulin and C-peptide. The samples were collected in sterilized tubes and kept at 4°C. After separating the clots, the serums were separated by centrifugation before insulin and C-peptide were measured by radio-immunoassay method.

Protein, triglyceride and urea determination: The total protein of serums was determined as per Lowry’s method (Lowry et al., 1951). Enzymatic methods were used to determine the concentrations of triglycerides (Chin and Abd El-Meguid, 1973). The blood urea nitrogen was measured using the diacetylmonoxime method (Friedman, 1953).
Statistical analysis: The data are expressed as Mean±SEM. The statistical analysis was performed using one way analysis of variance (ANOVA). The level of statistical significance was set at p<0.05. Calculations were performed using the SPSS statistical package.

RESULTS

Present results showed that induction of severe diabetes in rats by intraperitoneal injection of streptozotocin at 60 mg kg⁻¹ of b.wt. to suppress the secretion of insulin to the lowest level was necessary to avoid insulin interference with inhibitors. Table 1 shows that doses of streptozotocin at 60 mg kg⁻¹ of b.wt. cause 70 and 92% decrease in insulin and C-peptide production, respectively. This diabetic model of rat was found suitable for further study of hypoglycemic effect of phosphoenolpyruvate carboxykinase inhibitors. The in vitro effects of L and D Glutamic acids at 8 mM concentrations on phosphoenolpyruvate carboxykinase activity under optimum conditions are shown in Fig. 1. The inhibitory effects of glutamic acids are presented as remaining enzyme activity. The remaining activity of enzyme in the presence of L-glutamic acid was 11.92 nmol/min/mg, while that of D-glutamic acid was 11.41.

Table 1: Effect of induced diabetes with the dose of 60 mg kg⁻¹ of b.wt. streptozotocin on plasma level of insulin and C-peptide

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum insulin (mIU mL⁻¹)</th>
<th>Serum C-peptide (ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat (n=10)</td>
<td>2.0±0.2</td>
<td>0.62±0.003</td>
</tr>
<tr>
<td>Diabetic rat (n=10)</td>
<td>0.6±0.05</td>
<td>0.045±0.002</td>
</tr>
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The values are expressed as the Mean±SE p<0.05

The in vitro effect of different concentrations of D-glutamic acid on the enzyme activity is shown in Fig. 2. As depicted, at 12 mM concentration of D-glutamic acid, the enzyme activity was completely inhibited. Hill plots (inside curve) of the enzyme activity in the presence of D-glutamic acid show one binding site for D-glutamic acid and phosphoenolpyruvate carboxykinase. The curve interception shows that the inhibitory constant (K_i) of D-glutamic acid is 1.8×10⁻⁴ M.

The time course effects of L and D glutamic acids on blood glucose of diabetic and normal rats are shown in Fig. 3. Figure 3 shows that the D-glutamic acid causes a slight increase in blood glucose 1 h after injection. In fact, the total glucose concentration showed more than 10% increase at 0.5 h post-injection (data not shown). The inhibitory effect of D-glutamic acid began at 1.5 h post-injection and the decline of blood glucose continued for 4 h after injection, after which the glucose level began to increase slowly until it reaches its previous levels (data not shown). In the case of L-glutamic acid, the blood glucose increased with time till its depletion. It seemed, however, that gluconeogenesis was responsible for consuming L-glutamic acid as a precursor for glucose production, hence the increase of blood glucose.

Figure 4 summarizes the results of experiments done to study the inhibitory effects of D-glutamic acid on phosphoenolpyruvate carboxykinase activity. To this end a group of ten diabetic rats were injected intraperitoneally with doses of 100 mg kg⁻¹ b.wt., of D-glutamic acid dissolved in saline. Another group of ten diabetic rats were used as control and injected with the same volumes of saline buffer. After 2 h the animals were sacrificed and their livers extracted and homogenized before the enzyme activity.
Fig. 3: Alteration of glucose homeostasis in diabetic and not treated diabetic control rats following intraperitoneal injection of 100 mg kg⁻¹ of b.wt. of glutamic acid to diabetic 24 h fasted rats (n = 10) and same volume of saline to control 24 h diabetic rats (n = 10), respectively. The blood glucose determined by enzymatic method in samples obtained from the tail vein. The values are expressed as the Mean±SE p<0.05 for ten animals in each groups.

Fig. 4: In vivo determination of phosphoenolpyruvate carboxykinase activity after injection of 100 mg kg⁻¹ of b.wt. glutamic acid. Ten diabetic rats in treated group and ten diabetic rats in control group were chosen. Animals sacrificed 2 h after injection and the phosphoenolpyruvate carboxykinase extracted and its activity determined as described in methods. The values are expressed as the Mean±SE p<0.05.

activity was measured as described in method section. The data was pooled from 3 replicates of the same experiments.

The probable side effect of D-glutamic acid on metabolism of protein and lipids was also studied by measuring the total protein, urea level and triglyceride of blood samples after the injection of D-glutamic acid. Figure 5a-c depict the effect of D-glutamic acid on the serum total protein, on the blood urea and on the blood triglyceride, respectively. The figures indicate
no significant alteration in these parameters proving that D-glutamic acid has no side effect on the metabolism of protein and lipids.

DISCUSSION

In states of low carbohydrates diet or fasting, gluconeogenesis is the major source responsible for glucose homeostasis. Gluconeogenesis is a biochemical pathway by which glucose is synthesized from non carbohydrate precursors. The three regulatory enzymes in this pathway are phosphoenolpyruvate carboxykinase, Fructose-1,6-biphosphatase (FBPase) and glucose-6-phosphatase (Barthel and Schmoll, 2003). Phosphoenolpyruvate carboxykinase catalyzes the first rate limiting step of gluconeogenesis. In fed state, phosphoenolpyruvate carboxykinase biosynthesis is suppressed by insulin. The lack of insulin, due to its ceased biosynthesis or its receptors' dysfunction in diabetics, results in over production of gluconeogenic enzymes and hence leads to hyperglycemia. For several decades phosphoenolpyruvate carboxykinase inhibition has been and remains of great interest as a way to overcome diabetic hyperglycemia. Phosphoenolpyruvate carboxykinase, in contrast to other gluconeogenic enzymes plays a pivotal role in gluconeogenesis by making a metabolic sink for non carbohydrate intermediates entering the gluconeogenic pathway. This is why phosphoenolpyruvate carboxykinase inhibition is studied for drug design purposes. Amino acids as food constituents have different effects on glucose metabolism; some amino acid have hyperglycemic whereas some others have hypoglycemic effects. It is to mention that glutamic acid is used in food industry as monosodium glutamate to enhance the flavor of a wide variety of foods and generally recognized as safe additive by the FDA (Food and Drug Administration). However, there are reports which show that intake of L-glutamate causes obesity and diabetic like conditions in rat and mice (Matsuyama et al., 1973; Cameron et al., 1976; Nonaka and Oki, 1987). In a set of in vitro experiments using phosphoenolpyruvate carboxykinase substrate analogous, we found that L and D isomers of glutamic acids, having structures somehow similar to oxalacetate with four carbon skeleton and two carboxyl groups at the ends, produced significant inhibitory effects on isolated phosphoenolpyruvate carboxykinase activity. Figure 1 shows in vitro effects of 8 mM concentration of D-glutamic acid and L-glutamic acid on phosphoenolpyruvate carboxykinase in optimum conditions. The remaining activities of enzyme in the presence of these two amino acids are about 22% of the original activity. Figure 2 shows the Hill plot for D-glutamic acid. As shown in Fig. 2 (inside figure) the binding stoichiometry of glutamic acid per phosphoenolpyruvate carboxykinase is 1:1, which means that one D-glutamic acid binds to one phosphoenolpyruvate carboxykinase molecule and the binding constant of D-glutamic acid is $K_i = 1.8 \times 10^{-6}$ M. The same data were obtained for L-glutamic acid. The best doses for in vivo trials were obtained at 100 mg kg$^{-1}$ of animal body weight. Figure 3 shows the effects of i.p., injection of 100 mg kg$^{-1}$ b.wt. of D and L glutamic acids to groups of ten diabetic and control rats on their blood glucose. In accordance with the previous reports, our data in Fig. 3 shows that 100 mg kg$^{-1}$ b.wt. of L-glutamic acid causes rapid hyperglycemia as previously reported through oxidative deamination or transamination to oxaloacetate or pyruvate in liver (FAO, 1970, Cohen, 1939, Marcus and Reaven, 1967). As depicted in Fig. 3 hyperglycemic effects of L-glutamic acid begin after injection, reach its maximum at 3 hours post-injection and decline afterward; probably because of its depletion. In the case of D-glutamic acid, there is a significant increase in blood glucose after 0.5 h (data not shown) probably because of its conversion to oxaloacetate by D-amino acid oxidase thereby producing gluconeogenic precursor. It was shown that rat liver has only a poor ability to oxidise D-glutamic acid (Wilson and Koepp, 1961). We hypothesize that, this may be an explanation for the increase of inhibitory effects of D-glutamic acid and reduction of blood glucose after 1 h that continues for 4 h. However our data shows that at 4 h post-injection the blood glucose increased slowly and reaches its normal level after 5 h. To evaluate whether D-glutamic acts via phosphoenolpyruvate carboxykinase in decreasing blood glucose or not, the remaining activity of phosphoenolpyruvate carboxykinase in rat liver was determined at 2 h after injection in diabetic rats and compared to control groups. The enzyme inhibition was found in complete agreement with our postulated mechanism. While many side effects have been reported for L-glutamic acid, commercially known as monosodium glutamate as recently reported (Abeer Waggas, 2009), on central nervous system lesions, neuroendocrine disorders and typical symptoms like burning or heat sensation in the face, tightness or stiffness in the chest, arms and back, no side effects have been recognized for D-glutamic acid (Schaumburg et al., 1969, FAO, 1970). Our data on Fig. 5a-e shows D-glutamic acid has no significant effects on serum protein, urea and triglyceride and producing no change to their metabolism.
CONCLUSION

Our significant findings open new horizon for overcoming the diabetic hyperglycemia. Prolonged hypoglycemic effect of D-glutamic acid, besides having the same effect in flavoring as L-glutamic acid, its non toxic metabolic fate and unknown side effects make D-glutamic acid a good candidate for further pharmacological studies. This is emphasized by the fact that insulin resistance is exerted by over expression of phosphoenolpyruvate carboxykinase (Sun et al., 2002), therefore using D-glutamic acid may offer a solution in this context.

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