Insulin receptor tyrosine kinase activity in monocytes of type 2 diabetes mellitus patients receiving oral L-lysine

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The action of lysine as an antidiabetic agent was examined in human volunteers. Eight patients with type 2 DM were orally supplemented with L-lysine hydrochloride 1 g/day in two doses along with antidiabetic tablets (glyciphage or chlorformine), for a period of two months. Periodically their plasma fasting sugar and insulin receptor tyrosine kinase activity was measured in their monocytes. Eight normal healthy volunteers served as controls for comparison of receptor tyrosine kinase activity. Insulin receptor tyrosine kinase was isolated from monocytes by immunoprecipitation and the activity was determined using exogenous substrate poly glu-tyr (4:1) and radioactive ATP. Phosphorylated peptide was separated by electrophoresis and quantified using a liquid scintillation system. The enzyme activity was significantly low (22074±1728 dpm/ml immunoprecipitate) in subjects with diabetes when compared to non-diabetic control group (50,775 ± 3597). Lysine treatment enhanced the enzyme activity by 31% in patients with diabetes and decreased their blood sugar by 27%.

Insulin resistance in type 2 DM has been attributed to defects in insulin binding to its receptors and post receptor defects. It has been shown that tyrosine kinase activity of insulin receptor β subunit plays an important role in the insulin-induced signal transduction pathway. There are reports in which adipose tissue, fat, muscle and liver from individuals with type 2 DM have shown decreased tyrosine kinase activity of insulin receptors. Lysine and other amino acids have been reported to be beneficial in delaying cataractogenesis by their antiglycating effect. It has been reported that orally given lysine prevented cataract formation in experimentally induced diabetic rats. In addition to its being an antiglycating and anticataract agent, lysine was found to have antidiabetic effect as was seen by its ability to decrease blood sugar levels in diabetic rats and humans. In order to examine the mechanism by which lysine caused reduction in the blood sugar level, a pilot clinical trial of oral supplementation of L-lysine to type 2 DM subjects was undertaken. A novel mechanism involving improvement in insulin receptor tyrosine kinase activity in type 2 DM patients is proposed for the reduction in blood sugar level by lysine.

Materials and Methods

Ficoll hypaque, protamine, Na-orthovanadate and ATP were obtained from Sigma chemical company (USA). Heparin was procured from Biological Evans Ltd. (India). RPMI 1640 and penicillin were from High Media Ltd. (India) and antihuman insulin receptor antibody and agarose conjugate were purchased from Santa Cruz Biotechnology (CA, USA). Electrophoresis equipment and reagent supplies were from Bio-Rad (USA). [γ-32P] ATP with sp. activity 5000 Ci/mmol was procured from BRIT, (India). L-lysine hydrochloride tablets were from a pharmaceutical company Tablets India, Chennai, India. Other chemicals used were of analytical grade.

Subjects

Patients (type 2 DM) and controls were included in the study after getting written consent based on ethical committee recommendation of the institution. They were non-hypertensive, non-smoking and free from cardiac, kidney or ophthalmic disorders. They were on oral antidiabetic treatment such as glyciphage (500 mg/day) or chlorformine (5 mg). Average duration of diabetes in them was 12.7 ± 4 years. The control group comprised of non-diabetic healthy subjects free of any systemic diseases.
Study design

Eight type 2 DM patients were given 1.0 g/day L-lysine HCl tablets orally in two divided doses one each after breakfast and dinner along with their oral antidiabetic tablets. The dosage was fixed as 1.0 g L-lysine/day according to Kurupad et al.13 Patients were followed for two months periodically and biochemical estimations were conducted. Fasting blood sugar was estimated once a fortnight by o-toluidine method14. IRTK activity in monocytes was determined, by modifying the methods described earlier, initially and after lysine supplementation, i.e. at the end of each month for a period of two months15.16.

Isolation of monocytes

Peripheral blood (20 ml) was collected in heparin using 21G needle from the antecubital vein. Monocytes were separated by the Ficoll-hypaque technique17 and the cell pellet was free from platelets and erythrocytes. This cell fraction was mixed with equal volumes of RPMI 1640 (serum free), and incubated in a 25x25 mm² tissue culture plate at 37°C in 5% CO₂ for 4 hr. The cell homogeneity was examined using Nikon phase contrast microscope. The maintenance medium that had the floating lymphocytes (confirmed by Leishman staining18) was carefully aspirated and the adherent cells (monocytes) were used for immunoprecipitation of insulin receptor.

Immunoprecipitation of insulin receptor

The isolated monocytes were incubated with 3 ml of ice-cold RIPA buffer at 4°C for 10 min. After incubation 50 μl of 0.5% triton X 100 was added and the cells were disrupted by repeated aspiration through 21G needle at 4°C, followed by centrifugation at 4,000 rpm for 25 min at 4°C to pellet down cellular debris. The supernatant was transferred into a dry storage vial pre-cooled at 4°C. One ml of the supernatant was taken in a 1.5 ml microfuge tube and 10μl (2 μg) of anti-human insulin receptor antibody (insulin Rβ) was added and the tubes were incubated at 4°C for one hr. Next 20 μl of agarose conjugate was added and the tubes were incubated at 4°C for 12 hr in a rocker. The immunoprecipitate was collected by centrifuging the tubes at 2,500 rpm for 10 min at 4°C, dissolved in 1.0 ml of protein kinase assay buffer containing 50 mmol HEPES, 10 mmol EDTA and 0.15 M NaCl. This was stored in -20°C whenever not immediately used for the enzyme assay. Prior to the enzyme assay, the protein content of the immunoprecipitate was quantified by the method of Lowry et al.19, using bovine serum albumin as standard.

Tyrosine specific protein kinase activity of insulin receptor

The tyrosine specific protein kinase activity of insulin receptor was determined by modifying the method previously described15.16.20. The enzyme activity was determined using exogenous substrate poly Glu-Tyr (4:1) and [γ-32P] ATP. The phosphorylated substrate was resolved in 12% SDS PAGE and the gel was autoradiographed. The bands were excised from the gel and the amount of 32P incorporated into the substrate was measured using Beckman (LS 300) liquid scintillation system. Enzyme activity and sp. activity are expressed in terms of dpm/ml immunoprecipitate and activity / mg protein respectively.

Statistical analysis

Student 't' test (both paired and unpaired) to determine statistical significance was followed.

Results

Details of study subjects including age, sex, bodymass, initial blood sugar and blood sugar during lysine treatment are given in (Table 1). Total insulin receptor protein content, its tyrosine kinase activity and sp. activity in monocytes isolated from normal individuals and patients with diabetes are given in Table 2. All the eight patients with DM showed decrease in fasting blood sugar Table 1 as well as an increase in IRTK activity Table 2. The decrease in blood sugar was linear with period of treatment. At the end of two months, the mean decrease in fasting blood sugar value was 57 mg/dl (27%). There was also increase in insulin receptor protein Table 2, indicating that the net cellular protein synthesis is up-regulated with lysine supplementation. All the eight patients during treatment expressed a sense of well being. None showed any symptoms of hypoglycemia or adverse reaction or change in their body mass or blood pressure. Mild, but steady increase in sp. activity of IRTK during lysine treatment indicates that there is also a qualitative improvement in the receptor protein.
Table 1—Effect of lysine on the fasting blood levels in subjects with type 2 diabetes

[Patients were given lysine – HCl oral tablets 1.0 g/day along with other oral antidiabetic tablets]. Values are expressed as Mean ± SE

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Body mass (kg)</th>
<th>Fasting blood sugar (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43 ± 4.3</td>
<td>62.8 ± 2.8</td>
<td>75 ± 3.5</td>
</tr>
<tr>
<td>Diabetic group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Visit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50.8 ± 4</td>
<td>59.2 ± 2.7</td>
<td>211.8 ± 19</td>
</tr>
<tr>
<td>II Visit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>As above</td>
<td>As above</td>
<td>186.8 ± 20</td>
</tr>
<tr>
<td>III Visit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>As above</td>
<td>As above</td>
<td>155 ± 16.3*</td>
</tr>
</tbody>
</table>

*p < 0.01 (blood sugar between first and third visit in diabetic group)

Table 2—Comparison of tyrosine kinase activity of insulin receptor isolated from monocytes of normal and type 2 diabetic mellitus subjects

[All values are Mean ± SE from 8 patients and 8 controls]

<table>
<thead>
<tr>
<th>Protein concentration (μg/ml precipitate)</th>
<th>Total activity (dpm/ml of immunoprecipitate)</th>
<th>Sp. activity (×10^-3) units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1034 ± 108.5</td>
<td>50775 ± 3597</td>
</tr>
<tr>
<td>Diabetic group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Visit</td>
<td>564.5 ± 57.9**</td>
<td>22074 ± 1728**</td>
</tr>
<tr>
<td>II Visit</td>
<td>620.5 ± 57.3**</td>
<td>25889 ± 2203</td>
</tr>
<tr>
<td>III Visit</td>
<td>694.6 ± 79.2**</td>
<td>28845 ± 2313**</td>
</tr>
</tbody>
</table>

*p < 0.05, ** p < 0.01; *p < 0.01, enzyme activity when compared between control and diabetic group; p < 0.01, enzyme activity when compared between 1st visit and 3rd visit in diabetic group; p < 0.01, enzyme protein concentration when compared between control and diabetic group before lysine treatment; p < 0.05, enzyme protein concentration when compared between 1st visit and 3rd visit

Discussion

Insulin receptor protein was reported to be decreased in type 2 DM. This could be due to decreased number of receptors in circulating cells. We also observed similar findings (Table 2) suggesting that type 2 DM patients had both qualitative and quantitative defects in their insulin receptors. This could be due to the fact that amino acids can stimulate the total cellular/tissue protein synthesis. Khoanski reported that poly lysine induced insulin receptor tyrosine kinase activity and also its autophosphorylation. In another study Iritani et al. reported that dietary soybean protein, improved insulin receptor gene expression in rats.

In a recent pilot human clinical trial in a group of 20 type II DM subjects supplemented with oral lysine (1g/day), 12 patients showed a decline of more than 15% in blood sugar levels (both) fasting and postprandial. While their fasting insulin remained constant, PP insulin decreased indicating an improvement in the insulin sensitivity and decrease in insulin resistance. DM patients possibly have increased amounts of free amino acids due to decreased protein synthesis and after amino acid treatment their free amino acid levels decreased significantly suggesting that their cellular/tissue protein synthesis is augmented (manuscript in press).

Accumulation of non-enzymatic glycosylation products or AGE (advanced glycation end products) have been implicated as biochemical mechanism for the complications involved in ageing, DM (micro and macrovascular complications) and cardiovascular diseases. It is possible that insulin receptor itself might be glycated more in case of type 2 DM, than in normal which could result in insulin resistance in the form of post receptor defect. Lysine residue at position 1030 of insulin receptor β subunit is the ATP binding site, which plays an important role in insulin
receptor autophosphorylation and insulin mediated down stream signaling events. Chouck et al.\textsuperscript{22} have demonstrated that mutation in ATP binding site leads to insulin resistance characterized by decrease in the activity of IRTK. As lysine residue has greater chances of getting glycated in hyperglycemic environment, it could affect insulin mediated signal transduction pathway, by decreasing tyrosine kinase activity of its receptor. Thus, the exogenously supplemented lysine to type 2 DM subjects improved the IRTK activity in circulating mononuclear cells, probably by acting as an antiglycating agent.

Although amino acids have been shown to release insulin from pancreas\textsuperscript{21}, the release of insulin alone may not help patients with type 2 DM as most of them have severe insulin resistance\textsuperscript{4}. The improvement in the activity of IRTK and decrease in fasting blood sugar were not simultaneous, indicating that lysine can spare the insulin receptor protein lysine from getting glycated. Lysine may thus act as an antidiabetic agent by improving IRTK activity. However the extent of IR glycation in type 2 DM subjects needs to be investigated. Moreover the insulin induced signal transduction pathway is multifaceted, it being the network of multiple pathways like kinase cascades\textsuperscript{27}, IRS signaling system\textsuperscript{28} Ras activation/inactivation cycle\textsuperscript{29}, GLUT systems, protein-protein interactions and protein phosphatases signalling\textsuperscript{30} mechanisms. The effect of lysine in these pathways will be worth investigating to understand the role of lysine as an antidiabetic agent.

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References