Amino acids differentially regulate insulin receptor tyrosine kinase and phosphatidyl inositol-3-OH-kinase activities in human monocytes exposed to high glucose concentration

V Srinivasan1,2, M Rajesh1,5, K N Sulochana1,3, C Indra4 and S Ramakrishnan1*

1Biochemistry Research Dept, Vision Research Foundation, Sankara Nethralaya, 18 College Road, Chennai - 600 006, India
2Department of Cell and Molecular biology, Madras Diabetes Research Foundation, Chennai, India
3Department of Biological Sciences, National University of Singapore, Singapore
4Department of Cell Biology, Zoological Institute, Braunschweig, Germany
5National Heart Center & Johns Hopkins Singapore, Vascular Biology Laboratory, Republic of Singapore

Received 9 August 2004; revised 25 November 2004

Chronic hyperglycemia and insulin resistance are the common factors involved in the development of vascular complications in diabetes mellitus (DM) patients. Since insulin signaling pathway has been shown to be regulated by nutritional supplements, in the present study, we investigated the possible effects of free amino acids, such as lysine, arginine and alanine and their mixture in modulating the insulin receptor tyrosine kinase (IRTK) and phosphatidyl inositol-3-OH-kinase (PI3K) activities and on the changes in actin dynamics in monocytes (MC), exposed to high glucose concentration (25 mM). IRTK and PI3K activities were markedly decreased in MC, incubated with 25 mM glucose. However, on treatment with amino acids, only lysine was effective in augmenting IRTK and PI3K activities in a dose-dependent manner. Arginine had marginal effect in promoting these activities. Equimolar mixture of amino acids showed marginal effect of augmenting only IRTK activity. Alanine had no effect. The F-actin filaments showed grossly diminished organization in the cells treated with 25 mM glucose alone, as assessed by specific binding to phalloidin-FITC, when compared with cells treated with 5 mM glucose. On the other hand, a significant improvement in the F-actin organization was observed in the cells co-incubated with 25 mM glucose and lysine. A possible molecular mechanism is the antiglycating effect of amino acids. The signal transduction starts with binding of ATP to lysine at position 1030 in the & subunit of the receptor. This lysine (1030) may be protected by the added lysine or to some extent arginine from glycation and loss of function. In summary, our findings suggest that the amino acids apart from their antiglycating property can also modulate/influence the activities of pivotal enzymes that are upstream in the insulin-mediated signal transduction pathway and bring down glucose.

Keywords: Diabetes mellitus, insulin receptor tyrosine kinase, phosphatidyl inositol-3-OH-kinase, lysine and other amino acids, monocytes, glucose, insulin signaling pathway, actin dynamics, F-actin organization

IPC Code: C 12 Q 1/54

Diabetes mellitus (DM) represents a major health problem, which affects millions of people worldwide. The development of vascular complications is responsible for the morbidity and mortality in patients with DM. Although, a number of equally tenable hypothesis have been proposed for the development of vascular complications in DM, no single pathway has been pinpointed, implying thereby that complex mechanisms are involved in the pathogenesis of diabetic complications. Earlier, hyperglycemia and insulin resistance were reported to be the major factors responsible for the development of vascular complications in DM.

Insulin elicits its action by binding to its receptors on the target cell, leading to the autophosphorylation and increased tyrosine kinase activity in the cytoplasmic domain of the β-subunit, which in turn switches on various downstream signaling cascades. Insulin receptor tyrosine kinase (IRTK) and phosphatidylinositol-3-OH-kinase (PI3K) have been shown to be crucial regulators in this process. Increased IRTK and PI3K activities and GLUT4 effect increased internalization of glucose into the cell and...
oxidation to generate ATP. In addition, actin remodelling has also been shown to be the prerequisite for conformational change of insulin receptor (IR), which brings about the glucose uptake. IRTK and PI3K activities were found decreased in skeletal muscle obtained from diabetic patients. Also, structural impairment and diminished F-actin content have been documented in DM patients. Interestingly, insulin-mediated signal transduction is pivotal for glucose homeostasis, cell proliferation, differentiation, tissue development and growth. At molecular level, IR-mediated signal transduction is regulated by several factors, including nutrients/dietary supplements. Based on the insulin signaling, several drugs have been designed, which augment IRTK and PI3K activities or down-regulate aberrant expression of serine/threonine protein kinases. By activating IRTK and PI3K, insulin resistance is mitigated and there is better utilization of glucose inside the cells through oxidation.

Earlier, we reported that the free amino acids have antiglycating, anticyataractogenic and blood sugar lowering properties. In the present study, we investigated the effects of free amino acids, such as lysine, arginine and alanine and their mixture in modulating IRTK and PI3K activities in monocytes (MC), exposed to high glucose concentration (25 mM). MC were used, because the sampling is less invasive and has all the components of insulin signaling pathway. Moreover, we have earlier used MC to study IR signaling.

Materials and Methods

Special chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A), unless specified. [γ-32P] ATP with specific activity 5000 Ci/mmol was procured from Board of Radiation and Isotope Technology, Hyderabad, India. Antibiotic solution and RPMI 1640 medium were obtained from Himedia Laboratories, Mumbai, India, and liquid scintillation fluid was from SRL Laboratories, Mumbai. Protease inhibitor cocktail, antihuman insulin receptor (PI3K p85α) antibodies and protein A agarose conjugate were procured from Santa Cruz Biotech, CA, USA. Silica Gel 64 plate and common reagents (analytical grade) were obtained from E. Merck (Mumbai, India).

Subjects

Heparinized blood samples (10.0 ml) were collected from eight healthy male volunteers in the age groups 30-40 years, who were recruited from Vision Research Foundation, Chennai. Prior to blood collection, informed consent was obtained from all the subjects. The protocol involving human subjects strictly adhered to tenets of Helsinki declarations and was approved by the Institutional Research and Ethical Board.

Separation of monocytes

Heparinized blood, diluted with equal volumes of sterile PBS, pH 7.4 was carefully overlaid on equal volumes of ficoll-hypaque density gradient solution and centrifuged at 3000 rpm for 30 min in swing-out rotor at room temperature. Thereafter, the interface containing the mononuclear cells (lymphocytes and monocytes) was carefully and aseptically aspirated and washed thrice with PBS (pH 7.4), supplemented with antibiotic solutions. After final step of washing, the cell pellet was resuspended in serum-free RPMI 1640 medium and added to 100 mm tissue culture dish. The cells were allowed to adhere to the surface for 3 hr at 37°C in a 5% CO2 environment. After incubation, the floating cells were aspirated and discarded and the adherent cells were gently detached from the surface by washing the culture dish with sterile PBS. The adherent population contained at least 90-95% MC on microscopic examination after staining with Wright Giemsa stain. More than 95% of the cells were viable as determined by trypan blue exclusion test. MC separated from the individual subjects were pooled and used for further experiments. For each experiment unless specified, 2 x 10⁶ cells/ml were used and each experiment was performed in triplicates.

Treatment of cells

MC 2 x 10⁶/ml were either pre-incubated with physiological concentrations of glucose (5 mM) or 25 mM glucose in serum-free RPMI 1640 medium containing 100 nM insulin for 12 hr, followed by treatment with increasing concentrations of Lys, Ala, Arg (10-20 mM) or mixtures of these amino acids were added to the culture medium at concentrations of 10 mM, 15 mM and 20 mM, respectively along with 100 nM insulin at equal molar concentrations (1:1:1). After incubation for 12 hr, cells were harvested, washed twice with PBS and IRTK and PI3K activities were determined as described below. Total incubation period was 24 hr.

Materials and Methods

Special chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A), unless specified. [γ-32P] ATP with specific activity 5000 Ci/mmol was procured from Board of Radiation and Isotope Technology, Hyderabad, India. Antibiotic solution and RPMI 1640 medium were obtained from Himedia Laboratories, Mumbai, India, and liquid scintillation fluid was from SRL Laboratories, Mumbai. Protease inhibitor cocktail, antihuman insulin receptor (PI3K p85α) antibodies and protein A agarose conjugate were procured from Santa Cruz Biotech, CA, USA. Silica Gel 64 plate and common reagents (analytical grade) were obtained from E. Merck (Mumbai, India).

Subjects

Heparinized blood samples (10.0 ml) were collected from eight healthy male volunteers in the age groups 30-40 years, who were recruited from Vision Research Foundation, Chennai. Prior to blood collection, informed consent was obtained from all the subjects. The protocol involving human subjects strictly adhered to tenets of Helsinki declarations and was approved by the Institutional Research and Ethical Board.

Separation of monocytes

Heparinized blood, diluted with equal volumes of sterile PBS, pH 7.4 was carefully overlaid on equal volumes of ficoll-hypaque density gradient solution and centrifuged at 3000 rpm for 30 min in swing-out rotor at room temperature. Thereafter, the interface containing the mononuclear cells (lymphocytes and monocytes) was carefully and aseptically aspirated and washed thrice with PBS (pH 7.4), supplemented with antibiotic solutions. After final step of washing, the cell pellet was resuspended in serum-free RPMI 1640 medium and added to 100 mm tissue culture dish. The cells were allowed to adhere to the surface for 3 hr at 37°C in a 5% CO2 environment. After incubation, the floating cells were aspirated and discarded and the adherent cells were gently detached from the surface by washing the culture dish with sterile PBS. The adherent population contained at least 90-95% MC on microscopic examination after staining with Wright Giemsa stain. More than 95% of the cells were viable as determined by trypan blue exclusion test. MC separated from the individual subjects were pooled and used for further experiments. For each experiment unless specified, 2 x 10⁶ cells/ml were used and each experiment was performed in triplicates.

Treatment of cells

MC 2 x 10⁶/ml were either pre-incubated with physiological concentrations of glucose (5 mM) or 25 mM glucose in serum-free RPMI 1640 medium containing 100 nM insulin for 12 hr, followed by treatment with increasing concentrations of Lys, Ala, Arg (10-20 mM) or mixtures of these amino acids were added to the culture medium at concentrations of 10 mM, 15 mM and 20 mM, respectively along with 100 nM insulin at equal molar concentrations (1:1:1). After incubation for 12 hr, cells were harvested, washed twice with PBS and IRTK and PI3K activities were determined as described below. Total incubation period was 24 hr.
Immunoprecipitation of IRTK and PI3K

The cells were resuspended in 3.0 ml of ice-cold radio-immunoprecipitation assay buffer (RIPA), [PBS, pH 7.4, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 10 μl/ml protease inhibitor cocktail] at 4°C for 10 min. After incubation, 50 μl of 0.5% triton X 100 was added and cells were disrupted by repeated aspiration through 21G needle, followed by centrifugation at 4000 rpm for 25 min at 4°C to pellet down the cellular debris. Supernatant was transferred to a fresh micro-centrifuge tube, pre-cooled at 4°C and the tubes were incubated at 4°C for 1 hr. Following the above step, 20 μl of protein A agarose conjugate was added and the tubes were incubated at 4°C for 12 hr in a rocker. Subsequently, the tubes were centrifuged at 2500 rpm for 10 min at 4°C and immunoprecipitates (IP) were collected, supernatant was discarded and IP was washed thrice with RIPA buffer. Finally, IP were dissolved in 0.1 ml of protein kinase assay buffer containing 50 mM HEPES, 10 mM EDTA and 0.15 M NaCl and stored at -80°C, whenever not immediately used for the assay. Prior to freezing of IP, protein content was determined by Lowry method 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 as described. Briefly, isolated monocytes from the peripheral blood were treated with antihuman insulin receptor antibody. The immunoprecipitate was separated by centrifugation and tyrosine kinase 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 auto-radiographed and used as the template to identify the phosphorylated substrate in the gel. The bands were excised from the gel. The amount of [32P] incorporated into the substrate was measured using liquid scintillation system (Beckman LS 300 CA, USA). IRTK activity was expressed as pmol ATP incorporated/mM substrate/mg protein.

PI3K activity assay

The PI3K activity was determined by the method described earlier with slight modifications. Briefly, anti-p85α immunoprecipitates were washed 3 times with wash buffer 1 (1% nonidet P-40 and 100 mM sodium ortho-vanadate in PBS), thrice with wash buffer 2 (0.5 M LiCl, 0.1 M Tris, 100 μM sodium ortho-vanadate in water, pH 7.5) and twice with wash buffer 3 (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 100 μM sodium ortho-vanadate in water, pH 7.5). After final washing in buffer 3, PI3K-IP was incubated for 5 min with 50 μl wash buffer 3 in ice and PI3K activity in IP was determined as follows. Typically reaction-cocktail contained 20 μg substrate phosphatidyl inositol 3, 4-diphosphate (Sigma), 5 μg IP, 4 μl 50 mM ATP, and 2 μl 20 mM MgCl2. The reaction was initiated by adding 5 μl [γ-32P]-ATP (5 μCi/5 μl) and after 10 min of incubation at 37°C, was arrested by adding 20 μl of 8.0 M HCl. Total lipids were then extracted with 160 μl of chloroform:methanol (1:1) mixture. For separation of lipids, 50 μl of the extracted lower phase was applied on Silica gel 60 TLC plate pre-baked for 1 hr at 110°C. The plates were developed with chloroform:methanol:water: NH4OH (120:94:23:4). After separation, the plates were dried in fumehood and the lipids were stained with iodine vapours. Band corresponding to phosphorylated substrate was scrapped and transferred to 5.0 ml of liquid scintillant fluid and solubilized for 30 min at room temp. and radioactivity was measured using liquid scintillation system. PI3K activity was expressed as pmol ATP incorporated/mM substrate/mg protein.

Localization F-actin filaments

All experiments were performed in MC isolated from healthy subjects as described above. The cells were grown in glass cover-slips coated with 0.25% gelatin and their treatment was exactly the same as described in 'Materials and Methods'. After appropriate treatment, cells were washed with PBS, fixed in 4% formaldehyde for 20 min at room temp, rinsed in PBS and permeabilized using 0.5% triton X-100 for 10 min, followed by washing with two changes of PBS. Thereafter, cells were incubated with phalloidin-FITC (100 μg/ml, Sigma Chemicals) for 2 hr at room temp. in humid chamber protected from light. Phalloidin-FITC specifically binds to F-actin (polymerized and functionally active form of actin) and is useful in distinguishing F and G actins (monomeric form-inactive). After incubation, cover-slips containing cells were washed thoroughly v PBS, air-dried, mounted in microscopic slides.
Statistical analysis

Experiments were performed in triplicates and values were expressed as mean ± SD. One-way ANOVA, followed by Scheffe post-hoc test was used to evaluate the statistical fitness of the data. P < 0.05 was considered significant.

Results

IRTK and PI3K activities were markedly diminished (52% and 57% respectively), when MC were exposed to 25 mM glucose, when compared with cells incubated with physiological levels of glucose (Table 1). When MC were co-incubated with 25 mM glucose along with the free amino acids, such as Lys, Ala, Arg or their mixture in varying concentrations, characteristic variations in IRTK activity were observed. Lys was found to be effective in promoting IRTK activity in dose-dependent manner. However, Ala had no effect on IRTK activity, while Arg and equimolar mixture Lys, Ala and Arg exhibited marginal improvement in IRTK activity (Table 1).

Interesting results were obtained with PI3K activity, when MC were co-incubated with 25 mM glucose along with the free amino acids or their mixture. Lys showed improvement in PI3K activity in dose-dependent manner, with ~3-fold increase in MC co-incubated with 25 mM glucose and 20 mM Lys. However, Ala did not have any effect in restoration of PI3K activity in cells pre-incubated with 25 mM glucose. On the other hand, Arg at 20 mM exhibited marginal improvement in MC treated with 25 mM glucose (Table 1). However, mixture of free amino acids had little effect in improving PI3K activity.

Actin filaments play a crucial role in glucose homeostasis, by activation of insulin receptor and recruitment of glucose transporters to cell membrane. This culminates in efficient transport of glucose into the cells, where it undergoes oxidation to yield ATP. Any defect in actin organization would disrupt this process, resulting in insulin resistance, characterized by chronic hyperglycemia. Therefore, we investigated the effects of high glucose concentration on actin organization in MC obtained from healthy subjects and when MC were co-incubated with glucose and amino acids. In MC exposed to 5 mM glucose, gross presence of G-actin filaments, was observed, which was expected normally, since in resting cells, one would expect the presence of globular (G)-actin (Fig. 1A). However, when MC incubated with 25 mM glucose and 100 nM insulin alone for 12 h, no improvement in actin reorganization was observed i.e., conversion of G to F form was impaired (Fig. 1B). Normally, one would expect the predominance of F-actin filaments, when cells were activated with insulin and glucose. Persistence of G-actin in MC treated with insulin and high levels of glucose suggests that Insulin is not able to effect the conversion of G to F form. However, when MC were pre-incubated with 25 mM glucose + 100 nM insulin, followed by treatment with 20 mM Lys, abundance of F-actin filaments (fine streaks) was observed (Fig. 1C), suggesting that Lys promotes actin polymerization. On the other hand, MC pre-treated with 25 mM glucose + 100 nM insulin, followed by incubation with a mixture of equimolar (20 mM) concentrations of Lys, Ala and Arg revealed only globular

<table>
<thead>
<tr>
<th>Treatments (n=3)</th>
<th>IRTK activity</th>
<th>PI3K activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM glucose alone</td>
<td>8.53 ± 0.55</td>
<td>0.81 ± 0.07</td>
</tr>
<tr>
<td>25 mM glucose alone</td>
<td>4.51 ± 0.31***</td>
<td>0.35 ± 0.05*</td>
</tr>
<tr>
<td>25 mM glucose + 10 mM Lys</td>
<td>7.61 ± 0.36**</td>
<td>0.56 ± 0.08*</td>
</tr>
<tr>
<td>15 mM Lys</td>
<td>9.13 ± 0.21**</td>
<td>0.72 ± 0.06***</td>
</tr>
<tr>
<td>20 mM Lys</td>
<td>10.31 ± 0.61**</td>
<td>0.99 ± 0.11***</td>
</tr>
<tr>
<td>25 mM glucose + 10 mM Ala</td>
<td>3.97 ± 0.21</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>15 mM Ala</td>
<td>3.87 ± 0.22</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>20 mM Ala</td>
<td>4.53 ± 0.31</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>25 mM glucose + 10 mM Arg</td>
<td>4.81 ± 0.11</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>15 mM Arg</td>
<td>5.67 ± 0.32</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>20 mM Arg</td>
<td>6.57 ± 0.15*</td>
<td>0.61 ± 0.02**</td>
</tr>
<tr>
<td>25 mM glucose + 20 mM Mixture</td>
<td>6.05 ± 0.32*</td>
<td>0.44 ± 0.04</td>
</tr>
<tr>
<td>15 mM Mixture</td>
<td>6.11 ± 0.11*</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>20 mM Mixture</td>
<td>6.26 ± 0.47*</td>
<td>0.52 ± 0.04</td>
</tr>
</tbody>
</table>

* P<0.05 vs 25 mM glucose; ** P<0.01 vs 25 mM glucose; ***P<0.001 vs 25 mM glucose
Fig. 1—Effect of free amino acids on actin dynamics in MC in vitro as assessed by localization of actin filaments with phalloidin-FITC using fluorescence microscope [(A): MC incubated with 5 mM glucose alone. The presence of abundant G-actin filaments are seen; (B): MC were co-incubated with 25 mM glucose+100 nM insulin for 24 hr, followed by staining with phalloidin-FITC revealed the predominance of G-actin, suggesting impairment in actin organization; (C): actin organization in MC, pre-treated with 25 mM glucose+100 nM insulin for 12 hr followed by incubation with 20 mM Lys for 12 hr. The predominance of F-actin filaments may be seen as fine streaks (white arrow); and (D): actin organization in MC, pre-treated with 25 mM glucose+100 nM insulin for 12 hr followed by incubation with 20 mM mixture comprising of equimolecular concentrations (20 mM) of Lys, Arg and Ala. Actin organization was not improved much in this case, which could be seen by the presence of G-actin as patchy chunks (yellow arrow). All images were taken at x100. The Figure is representative of 2 independent experiments, yielding identical results] actin filaments seen as patchy dots (Fig. 1D).

Discussion

Insulin resistance in patients with type 2 DM is characterized by post-receptor defects, characterized by down-regulation or aberrant expression of membrane proximal or distal signaling proteins involved in insulin signaling pathway. Several mechanisms have been postulated for the post-receptor defects, which include polymorphism/mutation in genes that code for the downstream signaling proteins, aberrant expression of these proteins or glycation of proteins. Glycation of proteins has been implicated as an important mechanism for the development of vascular complications in DM. Inhibiting the formation of advanced glycation end (AGE) products like carboxymethyl lysine has been shown to ameliorate the vascular complications in DM. However, these studies have largely been limited to Ex vivo and animal models and little information is available on the human clinical trials. We have earlier reported that amino acids have the potential to prevent the formation of AGE and the development of cataract in animal model.

The disruption of actin filaments is reported to result in the inhibition of IRTK and PI3K activities and arrest the recruitment of glucose transporters to the plasma membrane, which results in improper glucose utilization. We have recently found that the incorporation of [U14C] glucose was lower in actin isolated from the leukocytes from diabetics, when compared with those from normal healthy subjects. Also, when the leukocytes from healthy subjects were incubated with high glucose concentration (25 mM), F-actin content was significantly reduced and the process was reversed on incubating the cells with amino acids.

Among the amino acids added to the cells incubated with high glucose concentration concentration (25 mM), only Lys was effective in augmenting IRTK and PI3K activities (Table 1), and in inducing actin polymerization in the cells. Arg had marginal effect in promoting these activities and on actin organization. Mixture of amino acids had marginal effect in augmenting IRTK activity only. Ala had no effect in either improving IRTK and PI3K activities or promoting actin organization. These observations suggest that individual amino acids behave differently in modulating the IR signaling pathway. Consistent with the observations from the present study, our pilot clinical trial earlier also showed that the supplementation of L-lysine to patients with type 2 DM, improved the IRTK activity and decreased fasting blood glucose levels. The differential action of the free amino acids in promoting IRTK and PI3K could be through their ability to induce the gene expression of transcription and regulatory elements, which control the expression of insulin-induced signaling proteins.

In conclusion, our findings suggest that apart from antiglycating property, amino acids also have the ability to modulate the key components of insulin
signaling pathway, which could help in glucose utilization and thereby reduction of blood glucose. One of the probable mechanisms is the protection of lysine at position 1030 of β subunit of insulin receptor from glycation and loss of receptor function. It is this lysine to which ATP binds and initiates the entire molecular events for glucose utilization and energy production.35,36 Once the lysine is shielded from glycation by glucose with the help of amino acids, it is free to accept ATP and trigger the molecular events for glucose utilization and energy production.37

Acknowledgement
The study was supported by grants from Vision Research Foundation, Chennai, India. The skillful assistance of Ms. K Parvathy Devi in preparing the manuscript and technical assistance from Ms. K Coral are gratefully acknowledged.

References
2 Kannel W B & McGee D L (1979) JAMA 241, 2035-2038
29 DeFronzo R A, Bonadonna R C & Ferrannini E I (1992) Diabetes Care 15, 318-368