REGULATION OF GLUCOSE TRANSPORTERS IN CELL MEMBRANE DURING DIABETES: IMPACT OF FLAXSEED OIL SUPPLEMENTATION

Jihan Hussein1*, Zakaria El-Khayat1, Olfat Shaker2, Dina Abo El-Matty3, Wafaa Rasheed1, and Jakleen Raafat1

1Medical Biochemistry Department, National Research Center, Doki, Giza, Egypt.
2Medical Biochemistry Department, Faculty of Medicine, Cairo University, Egypt.
3Biochemistry Department, Faculty of Pharmacy, Suez Canal University, Egypt.

ABSTRACT

Insulin resistance is associated with cell membrane properties. Some studies have demonstrated relationships between fatty acids composition of cell membrane phospholipids and insulin action. This study aimed to improve cell membrane structure (increasing the omega-3 fatty acids) in order to decrease insulin resistance through regulation of glucose transporters. Forty male albino rats were used in this study and divided into four groups. Group I (control group): healthy rats received a vehicle. Group II (flaxseed oil group): healthy rats received 1.2 ml flaxseed oil / kg b.w. / day orally. Group III (diabetic group): diabetic rats received a vehicle. Group IV (treated group): diabetic rats received 1.2 ml flaxseed oil / kg b.w. / day orally. Fasting blood sugar and plasma insulin were determined. Erythrocyte membrane fatty acids were estimated by HPLC column C 18 (260 X 4.6, particle size 5 μl), mobile phase was acetonitrile / water mixture (70/30) v/v by isocratic elution with flow rate 1 ml / min and 214 nm wave length. GLUT 1 and GLUT 4 were estimated by PCR. The current results indicated that, the percent of changes of GLUT1 and GLUT4 in treated group from diabetic were 18.42 and 44.64 % respectively; also a negative correlation was observed between insulin resistance and glucose transporters. In conclusion, flaxseed oil supplementation has an important role in improving the cell membrane structure and elevation of glucose transporters resulting in a reduction of insulin resistance during diabetes.

KEY WORDS: Cell membrane, glucose transporters, fatty acids, HPLC, insulin resistance.
INTRODUCTION
The earliest defect in developing type 2 diabetes is insulin resistance, characterized by decreased glucose transport and metabolism in muscle and adipocytes [1]. The transport of glucose into the myocardium is mediated by members of the facilitative glucose transporter (GLUT) family [2].

There are fourteen known members of the GLUT family: the Class I transporters GLUTs-1, -2, -3, -4, and -14, the class II transporters GLUTs-5, -7, -9, and -11, and the class III transporters GLUTs-6, -8, -10, -12, and HMIT [3,4]. These GLUT isoforms differ in their substrate specificity, kinetics of transport, and tissue distribution and localization. Many of the class II and Class III isoforms in the GLUT family have been discovered only in recent years, and the specific role that the newly identified GLUTs play in mediating the transport of hexoses across the membranes of mammalian cells remains poorly understood. The Class I transporters GLUT1 and GLUT4 are the most extensively studied GLUTs in mammalian tissues [2].

Of importance, the data suggest that the transport velocity of both GLUT1 and GLUT3 is limited only by environmental conditions (temperature, and pH) and degrees of cell surface expression greatly influence the rate of glucose uptake into cells [5]. Biophysical and structural studies indicate that interactions of membrane proteins with lipid molecules are critical to their folding and stability [6,7]. Changes in the phospholipid fatty acids composition of membranes will result in changes in the collective physicochemical properties of the bilayer, such as flexibility and fluidity. We therefore suggest that the fatty acid composition of membrane phospholipids is a cellular factor that may influence glucose transporters.

Great interest has been focused on the relationship between quality, not only the quantity, of dietary lipids and diabetic complications [8]. It was demonstrated that the type of dietary fatty acids influenced insulin sensitivity in adipocytes of sucrose-fed rats in different ways [9]. Thus, in this study we aimed to improve cell membrane structure (increase cell membrane omega-3 fatty acids) in order to decrease insulin resistance through regulation of glucose transporters (1 and 4).
MATERIALS AND METHODS

Materials
Streptozotosin (STZ) and fatty acids standards (HPLC) were purchased from Sigma Chemicals Co. (Munih, Germany). Flaxseed oil was purchased from the local market.

Experimental Animals
Forty male albino rats weighing 180-200 g were obtained from the animal house of the National Research Centre, Giza, Egypt. Standard laboratory food and water were provided ad libitum. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Methods
Induction of diabetes
STZ was dissolved in 50 mM sodium citrate solution (pH adjusted at 4.5) containing 150 mM NaCl. The solution containing (6.0 mg/100g body weight) was subcutaneously administrated in rats; fasting blood sugar was estimated after 3 days to confirm the development of diabetes mellitus [10].

Experimental design
Rats were divided into four groups of ten animals each. Group I (control group): healthy rats received a vehicle. Group II (flaxseed oil group): healthy rats received 1.2 ml flaxseed oil / kg b.w. / day orally. Group III (diabetic group): diabetic rats received a vehicle. Group IV (treated group): diabetic rats received 1.2 ml flaxseed oil / kg b.w. / day orally [11]. After the experimental period (8 weeks), animals were kept fasting for 12 hours before blood sampling, blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes and collected in tubes containing sodium fluoride for blood glucose estimation and heparinized tubes; 1 ml of heparinized blood was used for GLUT1 and GLUT4 determination by PCR and the remaining part was centrifuged at 4000 rpm for 10 minutes, plasma was separated and immediately frozen for estimation of plasma insulin. Packed RBCs were used for determination of cell membrane fatty acids by HPLC.

Biochemical analysis
Fasting blood sugar was determined using enzymatic colorimetric method [12]. Plasma insulin level was estimated by ELISA using BioSoure INS-EASIA Kit [13]. Insulin
resistance was calculated from the equation:

\[ \text{Insulin resistance} = \frac{\text{fasting glucose (mg dl}^{-1}) \times \text{fasting insulin (} \mu \text{IU ml}^{-1})}{405} \] [14].

**Fatty acids determination by HPLC**

**Erythrocyte membrane isolation**

The method used for erythrocyte ghost preparation is based on the hemolysis of RBCs in hypotonic solution for removal of hemoglobin [15], cell membrane was homogenized in 2% acetic acid- ethyl ether mixture (2:1 volume ratio). The solution was then filtered. The filtered suspensions were centrifuged at 500xg for 2 min., the organic and the aqueous phases were separated, the aqueous phase was shaken again with 2% acetic acid in ethyl ether (2:1 volume ratio) and the phases were separated [16]. The organic phases were combined and evaporated to dryness. The extract was dissolved in 200 ul acetonitrile [17].

**HPLC Condition**

This method was carried out after modification of the method described previously [11] using HPLC column C 18 (260 X 4.6, particle size 5 µl), mobile phase was acetonitrile / water mixture (70/30) v/v by isocratic elution with flow rate 1 ml / min and 214 nm wave length. Serial dilutions of standards were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

**Determination of Glut-1 and Glut-4 by PCR**

a) Total RNA extraction:

Total RNA was extracted using RNA extraction kit provided by Qiagene extraction kit. RNA purity and quantity was measured by spectrophotometer at 260 nm.

b) Primers sequence:

Five sets of primers were used for amplification of Glut-1 and Glut 4. All primers were supplied by Qiagene (Clinilab).

The primer sequence for Glut-1:

5'-GCCTGAGACCAGTGGAAAGCAC-3’
5'-CTGCTTAGGTAAAGTTACAGGAG-3’

The primer sequence for Glut-4:

F5-ACATACCTGACAGGGCAAGG-3
R5-CGCCCTTAGTTGGTCAGAAG-3
c) RT-PCR
Reverse transcription was carried out on 1 μg of total RNA, 0.25 μg random primers, 0.1 mM/L dNTPs mixture, 40 units of RNase inhibitor, 200 units of superscript II reverse transcriptase in 1 x PCR buffer (10 mM/L Tris-HCL, 1.5 mM/L MgCl₂ and 50 mM/L KCl, pH 8.3). The reaction was carried out at 37°C for 1 hr followed by 5 min. at 95°C to destroy the enzyme.

Quantitative real-time PCR for all genes expression were performed on the cDNA using the QuantiTect SYBR Green PCR Kit (Qiagen), according to the manufacturer's protocol. The PCR master mix was prepared by combining the following reagents to the final volume of 20:1 μl of sense primer (6.25 pmol/μl), 1 μl of antisense primer (6.25 pmol/μl), 10 μl of the enzyme dye mixture, and 8 μl of 1:16 cDNA. The PCR master mix was placed in a 96-well PCR plate following an initial denaturing step (95°C for 15 min), processed according to the following PCR protocol: denature 95°C for 30 s, anneal at 55°C for 30 s, and elongate at 72°C for 1 min for 39 cycles in a DNA Engine Option System (MJ Research, Alameda, CA). Plate read temperature was 80°C. The melting curve was performed from 65 to 95°C with reading every 0.2°C and holding for 5 s between reads. The final cooling temperature was set at 12°C. The data generated were analyzed by Opticon Monitor Software (MJ Research). Gene expression was normalized to GABDH expression level.

Statistical analysis
All results were expressed as mean ± S.E.; Data were analyzed by one way analysis of variance (ANOVA) SPSS. Ver.16 followed by LSD test to compare significance between groups. Difference was considered significant when P value ≤ 0.05.

RESULTS
In this study, fasting blood glucose and insulin resistance were significantly increased in diabetic group along with a significant reduction in insulin level compared to control, while these values improved by flaxseed oil administration in treated group (Table1). The current data indicated that, the ratio between omega3 and omega 6 also the ratio between omega3 and omega 9 in erythrocyte membrane were significantly decreased in diabetic group compared to control; whereas, these values were significantly increased by flaxseed oil administration in treated group compared to diabetic (table2).
Table (1): Blood glucose, plasma insulin and insulin resistance levels in the different studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose (mg/dl)</th>
<th>Insulin (µIU/ml)</th>
<th>Insulin resistance (mgdl⁻¹ µIU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Mean ± SE</td>
<td>76.60 ± 4.1</td>
<td>11.20 ± 0.9</td>
<td>2.12 ± 0.1</td>
</tr>
<tr>
<td>Flaxseed oil Mean ± SE</td>
<td>76.20 ± 2.7 b</td>
<td>11.50 ± 1.6</td>
<td>2.16 ± 0.1 b</td>
</tr>
<tr>
<td>Diabetic Mean ± SE</td>
<td>241.00 ± 9.3 a</td>
<td>9.10 ± 1.0 a</td>
<td>5.42 ± 0.2 a</td>
</tr>
<tr>
<td>Treated Mean ± SE</td>
<td>171.00 ± 5.1 a,b</td>
<td>10.20 ± 2.0</td>
<td>4.31 ± 0.1 a,b</td>
</tr>
</tbody>
</table>

Significant p value ≤ 0.05

a = significant difference compared to control group
b = significant difference compared to diabetic group
Number of cases = 10

Table (2): Erythrocyte membrane fatty acids in the different studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Omega-3 /omega 6 %</th>
<th>Omega-3 /omega 9 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Mean ± SE</td>
<td>1.18</td>
<td>5.5</td>
</tr>
<tr>
<td>Flaxseed oil Mean ± SE</td>
<td>3.28 ab</td>
<td>13.6 ab</td>
</tr>
<tr>
<td>Diabetic Mean ± SE</td>
<td>0.39 a</td>
<td>1.88 a</td>
</tr>
<tr>
<td>Treated Mean ± SE</td>
<td>1.93 b</td>
<td>6.42 b</td>
</tr>
</tbody>
</table>

Significant p value ≤ 0.05

a = significant difference compared to control group
b = significant difference compared to diabetic group
Number of cases = 10

In addition, glucose transporters were not changed in flaxseed oil group compared to control indicating the safety of flaxseed oil administration, while these values significantly decreased in diabetic group compared to control. However, flaxseed oil supplementation effectively increases the studied transporters (GLUT1 and GLUT 4) in treated group compared to diabetic group (table 3).
The percent of changes of GLUT1 and GLUT4 in diabetic group from control were -24% and -44% respectively. However, the percent of changes of GLUT1 and GLUT4 in treated group from diabetic were 18.42% and 44.64% respectively (fig. 1-4).

Table (3): Glut-1 and Glut-4 in the different studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glut-1 (RQ)</th>
<th>Glut-4 (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flaxseed oil</td>
<td>1.01 ± 0.39 b</td>
<td>1.01 ± 0.44 b</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.76 ± 0.04 a</td>
<td>0.56 ± 0.04 a</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>0.9 ± 0.04 a,b</td>
<td>0.81 ± 0.06 a,b</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant p value ≤ 0.0

a = significant difference compared to control group
b = significant difference compared to diabetic group

Number of cases = 10

Fig.(1) Percent of changes of Glut-1 in the different studied groups from control.
Fig.(2) Percent of change of Glut-1 in the treated group from diabetic group.

Fig.(3) Percent of changes of Glut-4 in the different studied groups from control.

Fig.(4) Percent of change of Glut-4 in the treated group from diabetic group.
In this study, we observed a negative correlation between insulin resistance and glucose transporters 1 and 4 in all studied groups (fig.5-6).

DISCUSSION
Insulin resistance is associated with cell membrane properties, some studies have demonstrated relationships between fatty acids composition of cell membrane phospholipids and insulin action [11].
In this study, the reduction of both ω3/ω6 and ω3/ω9 in the cell membrane in diabetic group may be resulting from the elevation of omega-6 and omega-9 which could be the result of enhanced lipid peroxidation in disease [18,19].

The reduction of omega-3 fatty acids and a shift from unsaturated towards saturated fatty acids content in the cell membrane phospholipid play a central, if not primary, role in increases the van der Waals forces between the hydrocarbon chains, reduce the membrane flexibility and causing more rigid arrays of phospholipid molecules in plasma membranes [20]. In addition, the increase in free fatty acids concentration results in an increase in intracellular fatty acyl-CoA (FAcyl CoA) and diacylglycerol (DAG) concentrations, results in activation of protein kinase C isoform (PKC-ε) leading to increase insulin receptor substrate-1 (IRS-1) serine phosphorylation. This in turn leads to decrease IRS-1 tyrosine phosphorylation and deacreas activation of IRS-1 associated phosphatidyleinositol 3-kinase (PI3-K) activity resulting in a reduction in glucose transporters (as was found in our experiment) and decreased insulin–stimulating glucose transport activity [21,22].

Flaxseed oil supplementation in the current study, significantly increased the ratio of omega-3 fatty acids to both omega-6 and omega-9 in the cell membrane which reflects the nature of flaxseed oil as a rich source of α-linolenic acid (omega-3). The most important result in this study was the reduction of insulin resistance along with glucose transporters (GLUT1 and GLUT4) elevation by flaxseed oil supplementation in treated group.

In agreement, Kato et al. [23]. indicated that the amounts of skeletal muscle GLUT-4 in α-linolenic acid treated mice was increased (250%) compared to control mice. In addition, Pifferi et al. [24] showed that endothelial Glut-1 significantly decreased (-23%) in the n-3 PUFA-deficient microvessels compared to control, whereas it increased (+35%) in the microvessels of rats fed the high n-3 PUFA diet. The study suggested that n-3 PUFA can modulate the brain glucose transport in endothelial cells of the blood-brain barrier, possibly via changes in Glut-1 protein expression and activity.

The improvement in glucose uptake after membrane enrichment with PUFA may be also related to an increase in the residency time of glucose transporter-4 (Glut-4) in the plasma membrane, which leads to an expansion of the intracellular pool of glucose-6-phosphate [25], and to increased skeletal muscle glycogen synthesis [26].
This increase in glucose transport activity in adipocytes was accompanied by both an increase in glucose transporter protein (Glut-4) and their mRNA levels. Also, Podolin et [25], recently demonstrated that the presence of fish oil (a rich source of omega-3 fatty acids) in the sucrose diet also prevented the development of the whole-body insulin resistance. The negative correlation that was observed in this study between insulin resistance and glucose transporters (1 and 4) appeared the role of glucose transporters in the reduction of insulin resistance and hence in the reduction of blood glucose level.

In conclusion, flaxseed oil supplementation significantly increases omega-3 fatty acids content in the plasma membrane resulting in elevation of glucose transporters that leads to the reduction of insulin resistance and blood glucose level during diabetes.

ACKNOWLEDGEMENT
Authors are grateful to the National Research Center, Giza, Egypt for unlimited help and support to carry out this work.

REFERENCES


which fatty acids inhibit insulin activation of insulin receptor substrat-1 (IRS-1)-associated phosphatidyleinositol 3-kinase activity in muscle. J. of Biological Chemistry, 2002; 277(52):50230-50236.


