PREVENTION OF PROTEIN KINASE C ISOZYMES ELEVATION BY FLAXSEED OIL IN EXPERIMENTAL DIABETES

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ABSTRACT

Protein kinase C (PKC) is a key enzyme in insulin action and the activation of PKC isoforms prevents phosphoinositide 3-kinase (PI3K) pathway at the insulin receptor substrate level, which affects several signaling molecules related to this pathway. Arachidonic acid (AA) and oleic acid (OA) are thought to stimulate PKC isoforms activity through direct interactions with binding sites in the regulatory domain of the enzyme. The main objective of this study was to evaluate the role of flaxseed oil supplementation in prevention of protein kinase C isozymes elevation in experimental diabetes and then its role in reducing insulin resistance. Sixty male albino rats were used in this study and divided into four groups: control, flaxseed oil, diabetic and treated groups. After the experimental period (8 weeks), fasting blood sugar and insulin were estimated. Erythrocyte membrane AA and α linolenic acid (ALA) were determined 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 200 nm wave length. PKC βII and PKC γ were estimated by PCR. PKC βII, PKC γ and AA were significantly increased in diabetic group while flaxseed oil supplementation significantly decreased these values in treated group. In conclusion, flaxseed oil supplementation increased erythrocyte membrane ALA and decreased AA resulting in a prevention of PKC isozymes elevation and improving insulin sensitivity in experimental diabetes.

Key words: PKC, fatty acids, flaxseed oil, insulin resistance, HPLC.
INTRODUCTION

Diabetes mellitus, is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced\(^1\), it is characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, action or both\(^2\).

Protein kinase C (PKC), a key enzyme in insulin action is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes play important roles in several signal transduction cascades\(^3\).

The PKC family consists of fifteen isoforms; they are divided into three subfamilies: conventional (or classical), novel, and atypical. Conventional PKCs contain the isoforms \(\alpha\), \(\beta I\), \(\beta II\), and \(\gamma\). Novel PKCs include the \(\delta\), \(\varepsilon\), \(\eta\), and \(\theta\) isoforms. On the other hand, atypical PKCs (including protein kinase \(\zeta \) and \(\iota / \lambda\) isoforms). The term "protein kinase C" usually refers to the entire family of isoforms\(^4\).

A previous study observed that chronic feeding of oleic acid (OA) / \(\omega – 6\) fatty acid – enriched diet (19% corn oil ) increase endogenous PKC activity in the mouse hypothalamus\(^5\). Also, arachidonic acid (AA) has been shown to activate conventional, novel, and typical PKC isoforms\(^6\). Interestingly, the lipooxygenase metabolites of arachidonic acid, lipoxin A, and 12-HETE, also stimulate PKC isozyme activity\(^7\). Contrarily, it was found that, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (\(\omega -3\) fatty acids) exert a net inhibitory effect on PKC isozymes activity\(^8\).

From this point of view we aimed to study the role of flaxseed oil supplementation (as a source of \(\omega -3\) fatty acids) in attenuating the elevation of protein kinase C isoforms and hence in reducing insulin resistance in experimental diabetes.

MATERIALS AND METHODS

Materials

Streptozotosin (STZ) was purchased from Sigma Chemicals Co. (Munih, Germany). \(\alpha\)-linolenic acid (ALA) and arachidonic acid (AA) standards (HPLC grade) were purchased from Sigma Chemicals Co. (Munih, Germany).
Experimental Animals
Sixty male albino rats weighing 180-200 g were obtained from the animal house of National Research Center, Giza, Egypt., and fed a standard commercial diet (control diet) purchased from the Egyptian company of oils and soaps. Water was available ad-libitum for acclimatization before starting the experiment, kept under constant environmental conditions at room temperature. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre (NRC).

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 [9].

Experimental design
Sixty male albino rats were used in this study and divided into the following 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 [10].
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:

1- Tubes containing sodium fluoride for blood glucose estimation.
2- Heparinized tubes for estimation of other biochemical parameters as follow:
   • 1 ml whole blood was used for PKC βII and PKC γ determination by PCR.
   • The remaining part was used for estimation of erythrocyte membrane fatty acids (ALA and AA) ; this part was centrifuged at 4000 rpm for 10 minutes, plasma was separated and immediately frozen. Packed RBCs were used for isolation of erythrocyte membrane to estimate erythrocyte membrane fatty acids by HPLC.
Fasting blood sugar was determined using enzymatic colorimetric method \[11\]; Centronic, Germany.

Plasma insulin level was estimated by ELISA using BioSoure INS-EASIA Kit\[12\].

Insulin resistance was calculated from the equation

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

Analysis of cell membrane fatty acids by HPLC

Cell membrane was homogenized in 2 % acetic acid-ethyl ether mixture (2:1 volume ratio). The solution was then filtered and centrifuged at 500 xg, the organic phase was evaporated to dryness. The extract was dissolved in 400 ul acetonitrile to be ready for injection in HPLC \[14\].

HPLC Condition

This method was carried out after modification of the method described previously \[15\]. HPLC column C 18 (250 × 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 200 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 concentrations in samples were obtained from the curve.

Determination of PKC βII and PKC γ by PCR

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.

Primers sequence

Five sets of primers were used for amplification of PKC BII and PKC γ. All primers were supplied by Qiagene. PKC βII and PKC γ sequences were bought as ready made kits from QIAGEN.

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 Quanti Tect 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 and 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
Results were expressed as mean ± standard error. Data were analyzed by independent sample t test (SPSS) version 15 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 levels were elevated in diabetic group compared to control, while these values decreased by flaxseed oil administration in treated group (Table1). The mean value levels of protein kinase C isoforms (PKC βII and PKC γ) were significantly increased in diabetic group compared to control while these values decreased again by flaxseed oil administration in treated group compared to diabetic (table2). In this study flaxseed oil administration caused a significant elevation in α-linolenic acid concomitant with a significant decrease in arachidonic acid levels compared to control. In diabetic rats, there was a significant decrease in α-linolenic acid and a significant increase in arachidonic acid were observed (Fig.1,2). However, administration of flaxseed oil improved these values, thus α-linolenic acid was significantly increased in treated group compared to the diabetic one to reach the values of the normal group, in addition, arachidonic acid was significantly decreased in treated group compared to diabetic, although it was still significantly increased compared to control (Fig.1,2).
Table 1: Blood glucose, insulin and insulin resistance levels in 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>79.9 ± 4.1</td>
<td>11.3 ± 0.9</td>
<td>2.22 ± 0.1</td>
</tr>
<tr>
<td>Flaxseed oil Mean ± SE</td>
<td>79.6 ± 2.7</td>
<td>11.6 ± 1.6</td>
<td>2.27 ± 0.1 b</td>
</tr>
<tr>
<td>Diabetic Mean ± SE</td>
<td>245 ± 9.3 a</td>
<td>8.9 ± 1.0 a</td>
<td>5.38 ± 0.2 a</td>
</tr>
<tr>
<td>Treated Mean ± SE</td>
<td>176 ± 5.1 a,b</td>
<td>10.5 ± 2.0</td>
<td>4.56 ± 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 = 15

Table 2: PKC βII and PKC γ in different studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>PKC βII (RQ)</th>
<th>PKC γ (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Mean ± SE</td>
<td>1 ± 0.03</td>
<td>1 ± 0.03</td>
</tr>
<tr>
<td>Flaxseed oil Mean ± SE</td>
<td>0.85 ± 0.02 a,b</td>
<td>0.93 ± 0.03 b</td>
</tr>
<tr>
<td>Diabetic Mean ± SE</td>
<td>1.56 ± 0.04 a</td>
<td>1.48 ± 0.03 a</td>
</tr>
<tr>
<td>Treated Mean ± SE</td>
<td>1.15 ± 0.02 a,b</td>
<td>1.2 ± 0.03 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 = 15
Positive correlation was observed between insulin resistance and protein kinase C (PKC βII & PKC γ) and arachidonic acid (Fig. 3-5), although negative correlation was observed between insulin resistance and α-Linolenic acid (Fig. 6).

**Fig. 1**: α-Linolenic acid levels in different studied groups

**Fig. 2**: Arachidonic acid levels in different studied groups

**Fig. 3**: Correlation between insulin resistance and PKCβII
Fig. 4 Correlation between insulin resistance and PKCγ

Fig. 5 Correlation between insulin resistance and arachidonic acid.

Fig. 6 Correlation between insulin resistance and α-linolenic acid.
DISCUSSION

The stimulation of PKC by fatty acids has been well characterized\textsuperscript{[16]}. For example, saturated fatty acids that have carbon chain lengths of C13 to C18 activate PKC-\(\gamma\) in vitro\textsuperscript{[17]}. The change that was observed in AA in this study in diabetic group may be due to the enhanced lipid peroxidation resulting from diabetes\textsuperscript{[18]}. Alternatively, the increased AA levels could be due to elevated desaturase activity on linoleic acid (LA) leading to increased formation of prostaglandins and other lipoxygenase products\textsuperscript{[19]}. These values of fatty acids were regulated in our experiment by flaxseed oil administration probably due to its high content of omega-3\textsuperscript{[18]}.

In this study, PKC \(\beta\) II and PKC \(\gamma\) significantly increased in diabetic group compared to control. This result was in agreement with the previous study\textsuperscript{[20]} which indicated that, in kidney biopsies of diabetic patients, quantitative real-time PCR analysis showed a 9.9-fold increase in PKC-\(\beta\) mRNA expression as compared to control subjects. It was suggested that PKC activation, especially the \(\beta\) isoform induced by hyperglycemia, may be responsible for the endothelial dysfunction observed in diabetic patients\textsuperscript{[21]}.

In addition, Shirai et al.\textsuperscript{[6]} indicated that, arachidonic acid (AA), the principal polyunsaturated \(\omega\)-6 fatty acid in the cell membrane in diabetic patients, increase PKC activity in a concentration- dependant manner in the absence of calcium, diacylglycerol (DAG), and phosphatidylserine. AA, has been shown to activate conventional, novel and a typical PKC isozymes\textsuperscript{[22]}. Interestingly, the lipoxygenase metabolites of arachidonic acid also stimulate PKC isozyme activity\textsuperscript{[7]}. In contrast to the stimulating effects of arachidonic acid, the omega-3 fatty acids appear to inhibit PKC activity\textsuperscript{[23]} as was found in the current study; thus, flaxseed oil (a rich source of \(\alpha\) linolenic acid) administration significantly decreased PKC –\(\beta\) II and \(\gamma\) in treated group to become more or less near the normal range.

The increase in AA concentration in the cell membrane results in an increase in intracellular fatty acyl-CoA and diacylglycerol (DAG) concentration, which results in activation of PKC isoform leading to increased insulin receptor substrate -1 (IRS-1) serine phosphorylation; this in turn leads to decreased IRS-1 tyrosine phosphorylation and decreased activation of IRS-1 associated phosphatidylinositol 3-kinase (PI3-K) activity resulting in decreased insulin-stimulating glucose transporter activity\textsuperscript{[24]}. Thus, saturated fatty acids induced phosphorylated PKC isoform is involved in the inhibition of (IRs) gene transcription by
impairing architectural transcription factor of IRs gene. This transcription factor is a group of high mobility protein (HAMGA 1) that reduces (IRs) protein on cell membrane [25].

The positive correlation in this study between insulin resistance and AA, PKC–β II and PKC–γ was supported by Geraldes and King [21], who indicated that elevation of cell membrane AA increased PKC-induced insulin signaling inhibition and increased insulin resistance. He suggested that, PKC activation prevents PI3K pathway at the insulin receptor substrate level, which affects several signaling molecules related to this pathway.

We concluded that, flaxseed oil supplementation effectively prevents PKC isozymes elevation resulting in a reduction in insulin resistance during diabetes.

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