Quercetin and epigallocatechin gallate effect on the lipid order parameter of peripheral blood mononuclear cells from diabetes patients

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Abstract

The study aimed at in vitro effects evaluation of vegetal antioxidants (quercetin – Q and epigallocatechin gallate – EGCG) on plasma membrane properties of peripheral blood mononuclear cells (PBMC) isolated from diabetes patients. A number 13 type II diabetes mellitus (T2DM) and 8 type I diabetes mellitus (T1DM) patients were selected and compared them 21 healthy control subjects. The protocol was approved by the local ethics committee and the informed consent of the patients was obtained. The PBMCs were separated from à jeun blood samples drawn from the subjects, treated with the above mentioned antioxidants at room temperature for 20 minutes, and evaluated for their membrane incorporated TMA-DPH fluorescence anisotropy (r) and the fluidity (f) using data gathered in steady state fluorescence polarization measurements.

Q (1 µM) increased the membrane fluidity for 64.7% in control subjects and 27.3% of T2DM patients; EGCG (10 µM) increased the membrane fluidity in 57.1% controls and 44.4% T2DM patients. Both Q and EGCG had a positive and stable in vitro effect on the membrane fluidity of healthy subjects. For diabetes patients, a certain damage of the cell membrane could be putted into evidence.

Keywords: fluorescence anisotropy, peripheral blood mononuclear cells, diabetes mellitus

Introduction

The modern view of a cell membrane is that of a cooperative system consisting in a phospholipid bilayer with the polar head groups facing the inner and outer sides of the cell, in which molecules such as proteins and cholesterol are embedded [1]. Considerable evidence has been accumulated for the important role of the plasma membrane structure in signal transduction and other cellular processes. Membrane structural heterogeneity is likely to be particularly important for enzymes that have lipids as substrates, including phospholipases. For example, it is well established that impairments in the membrane bilayer modulate the phospholipase A2 activity, enzyme that generates important lipid second messengers, as well as amphiphilic products that can modulate the membrane structure and influence processes such as vesicle budding and exocytosis [2].
Plasma membranes are lipoprotein complexes and the maintenance of lipid micro-domains fluidity is prerequisite for the functions, viability, growth and reproduction of the cells. Membrane fluidity correlates with the rotational and lateral diffusion rates of the membrane components. In the absence of other constraints, most lipids and unrestrained integral proteins freely diffuse in the plane of the membrane with high diffusion coefficients. Membrane fluidity was shown to have a decisive role in the efficiency of ligand binding, in the outcome of direct cell to cell contacts and in the modulation of the membrane enzymes activity, being dependent only on the mobility freedom of the membrane constituents. It is nowadays known that the increased release of free radicals and reactive oxygen species (ROS) affects the membrane fluidity, affects the cellular Ca$^{2+}$ homeostasis and induces lipid peroxidation leading finally to the cell death. Membrane fluidity changes have been described in thrombocytahemia, hyperlipidaemia, hypercholesterolaemia, hypertension, diabetes mellitus, obesity, septic conditions, in allergic and burnt patients, in alcoholics, in Alzheimer's disease, in schizophrenia, etc [3].

Diabetes mellitus is a complex metabolic disorder characterized by pathological changes of glucidic, lipid and protein metabolism due to chronic hyperglycemia. Several links relate the cellular metabolism and chronic hyperglycemia. Among them the ATP synthesis by oxidative phosphorylation and cellular energy metabolism (ATP/ADP ratio), redox status and reactive oxygen species (ROS) production; membrane potential and substrate transport across the mitochondrial membrane are involved in different steps of the very complex glucose metabolism network. Recently, it has been pointed out that mitochondrial ROS production is essential in the signaling pathway of harmful effects produced by hyperglycemia. Thus, increased production of free radicals is associated with the physiopathology of diabetes, resulting in oxidative damage to lipids and proteins [4,5,6].

This paper presents preliminary results of the in vitro effect of quercetin (Q) and epigallocatechin gallate (EGCG) on the cell membrane of PBMC obtained from type I and II diabetes patients compared to healthy controls by means of evaluation of fluorescence anisotropy of the membrane incorporated probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) following steady-state fluorescence intensity measurements.

Materials and methods

Study design
We selected 42 subjects (40 to 60 years old) as following:
- 13 patients with type II diabetes mellitus (T2DM group)
- 8 with type I diabetes mellitus (T1DM group)
- 21 normoglycemic, normolipidemic patients that constituted the control group.

The diagnosis of diabetes (either T1DM or T2DM) was based on current World Health Organization criteria. Patients with severe renal, hepatic or hematological disease, overt cardio-vascular disease or malignancy were excluded from the study. None of the subjects had taken known antioxidants containing supplements (vitamin C, vitamin E, probucol, etc). The studied patients had no evidence of vascular complications, including hypertension or coronary artery disease. Controls were defined as not having a major medical illness, no previous hospital admissions, no current medication, and a subjective perception of good health as determined by health questionnaire.
Informed consent was obtained from each subject participating in the study. The protocol was approved by the local ethics committee.

Using á jeun venous blood samples we evaluated for all the subjects the TMA-DPH fluorescence anisotropy and membrane fluidity of the untreated peripheral blood mononuclear cells (PBMC). Each sample of PBMC was incubated for 20 minutes at 25°C with Q (1µM) and EGCG (10 µM); following the incubation we evaluated again the fluorescence anisotropy and the fluidity of the cell membranes.

Materials

Biologic material: 5 mL of peripheral venous blood was sampled on EDTA-Na₂ as anticoagulant.

Reagents: Hystopaque 1077 was purchased from Merck, RPMI 1640 medium was purchased from Biochrom AG, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-p-toluenesulfonate (TMA-DPH) was obtained from Molecular Probes.

Devices: LS50 B spectrofluorimeter (Perkin Elmer), equipped with thermostated cell holder, magnetic stirring and fluorescence polarization accessory.

Sample preparation

Venous á jeun blood samples were drawn from the patients. The separation of the cells was performed using the density gradient method; the samples were centrifuged at 600 g for 20 minutes, at 25°C using Hystopaque 1077. The plasma samples obtained after the separation of the cells were used for the evaluation of the glucose level (with commercial glucose-oxidase kits).

The PBMC cell ring obtained, containing mainly lymphocytes and monocytes, was suspended in RPMI 1640 medium supplemented with sodium bicarbonate and L-glutamine, and the suspension was centrifuged at 450 g, for 10 minutes, at 4°C. The PBMC pellet was collected, re-suspended in RPMI 1640 medium, and once again washed with RPMI medium (210 g, 10 min, 4°C). The supernatant was discarded, the pellet was resuspended in 3 mL of RPMI 1640 medium and the cells were counted in a Burker-Türk chamber. To be used in fluorescence anisotropy measurements, the PBMC suspension was standardized at 10⁵ cells/mL with RPMI 1640 medium.

Fluorescence anisotropy evaluation

The fluorescence anisotropy of TMA-DPH incorporated in PBMC was assessed by the determination of steady state fluorescence polarization of the membrane-fluorescent probe system; the probe (TMA-DPH) lacks fluorescence in solution and becomes fluorescent when incorporated into the lipid membrane bilayer.

We evaluated the autofluorescence of each sample of normalized PBMC using a Perkin Elmer LS50B spectrofluorimeter. For the measurement of the changes in the TMA-DPH fluorescent properties following the membrane insertion, we added to a 2 mL of normalized PBMC suspension an aliquot of TMA-DPH stock solution in DMSO to get a 2.5 µM TMA-DPH in the measurement cuvette. The cell suspension with the fluorescent probe was incubated for 2 minutes at 37°C under continuous magnetic stirring. Steady state fluorescent polarization of TMA-DPH was further measured; the TMA-DPH was excited with polarized light at 340 nm and the emission intensities were detected at 425 nm, through another polarizer system.

We incubated each sample with Q (1 µM) and respectively EGCG (10 µM) for 20 minutes at room temperature, and then we measured again the TMA-DPH fluorescent properties.

Calculation of the fluorescence anisotropy (r) was performed according to the equations (1) and (2):
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\[ r = \frac{I_{vv} - G I_{vh}}{I_{vv} + 2G I_{vh}} \]  \hspace{1cm} (1)

\[ G = \frac{I_{hv}}{I_{hh}} \]  \hspace{1cm} (2)

where \( r \) is the fluorescence anisotropy, \( I_{vv}, I_{vh}, I_{hv} \) and \( I_{hh} \) represent the emission intensity corrected for the autofluorescence signal of unstained cells, when the polarizers in the excitation and emission beams are oriented in vertical-vertical, vertical-horizontal, horizontal-vertical and horizontal-horizontal positions, respectively [7, 8].

The lipid order parameter in the polar head-group region (S) was computed following equation (3), using the limiting initial \( r_0 \) and long-time \( r_\infty \) values of the fluorescence anisotropy of TMA-DPH-membrane complex [9, 10, 11]:

\[ S = \sqrt{\frac{r_\infty}{r_0}} \]  \hspace{1cm} (3)

where \( r_\infty = 1.270 \cdot r - 0.076 \) for \( 0 < r < 0.28 \) and \( r_\infty = 1.100 \cdot r - 0.032 \) for \( 0.28 < r < 0.34 \) and \( r_0 = 0.362 \).

The membrane fluidity \( f \), was considered as the reciprocal of the lipid order parameter S.

As for most of the T1DM patients the anisotropy of the complex TMA-DPH could not be computed because the \( r \) value was higher than the limiting initial \( r_0 \) value of the probe itself, we assumed that the membrane did not incorporate the probe and we computed a membrane apparent binding parameter (P), following equation (4):

\[ P(\%) = \frac{I_{DPH}_{vv} - I_{0vv}}{I_{0vv}} \times 100 \]  \hspace{1cm} (4)

where \( I_{DPH}_{vv} \) represents the intensity of the vertical-vertical fluorescence emission of the cells incubated with TMA-DPH and \( I_{0vv} \) represents the intensity of the vertical-vertical autofluorescence emission of the PBMC’s lacking the fluorescent probe.

Statistical analysis

Results are expressed as means±standard deviation (SD). Statistical analyses were performed using the Student t test and differences were considered significant for \( p < 0.05 \).

Results and discussion

Hyperglycemia associated with diabetes mellitus determines oxidative stress that leads to important functional and biochemical changes of the endothelial of the blood cells as well. Previous studies showed that polymorphonuclears from diabetic subjects have been found to be abnormal in various functional activities mediated by the plasma membrane. The alterations of the physicochemical properties of the plasma membrane could be the basis of the modifications in functional activities of polymorphonuclears and could be the result of metabolic and chemical modification associated with type I and type II diabetes mellitus [12, 13].

The fasting plasma glucose was significantly higher (\( p < 0.0001 \)) for both T2DM patients compared to T1DM and to controls (Table 1).
Table 1. The obtained values for the fasting plasma glucose and studied biophysical parameters of the PBMC membrane

<table>
<thead>
<tr>
<th>Evaluated parameter</th>
<th>Control group</th>
<th>Type II diabetes mellitus group</th>
<th>Type I diabetes mellitus group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mol/L)</td>
<td>85.80±7.25</td>
<td>149.78±23.65</td>
<td>259.50±74.61</td>
</tr>
<tr>
<td>Fluorescence anisotropy</td>
<td>0.28±0.03</td>
<td>0.24±0.05</td>
<td>0.28±0.03*</td>
</tr>
<tr>
<td>Lipid order parameter</td>
<td>0.88±0.06</td>
<td>0.78±0.11</td>
<td>0.87±0.08*</td>
</tr>
<tr>
<td>Membrane fluidity</td>
<td>1.14±0.08</td>
<td>1.30±0.17</td>
<td>1.14±0.11*</td>
</tr>
</tbody>
</table>

*the values correspond to 25% of the T1DM group, for which the fluorescence anisotropy could be calculated

The fluorescence anisotropy of untreated cells was largely equal (Table 1, Figure 1). The T1DM group was heterogeneous concerning the insertion of the fluorescent probe. For most of the patients, we noticed that the calculated fluorescence anisotropy parameters were either negative, or exceeded the limiting value of the molecular reporter (0.362 for TMA-DPH), suggesting unincorporation of the probe, confirmed by comparison of the TMA-DPH fluorescence and the unstained cells autofluorescence intensity values. The experimental data allowed the calculation of r only for 25% of the T1DM patients.

![Figure 1. Baseline membrane fluidity determined for the groups](image)

The computed membrane fluidity of unexposed to antioxidants cells belonging to the groups was largely equal. To be noticed that only for 25% of the T1DM group the membrane fluidity could be actually computed (see text)
Figure 2. Baseline apparent binding parameter determined for the groups. The membrane of the cells from the groups was unequally permeable to the insertion of the fluorescent probe (TMA-DPH), suggesting unimpairments in normal cell functions and/or structure.

Figure 3. Effect of the PBMC chemical exposure to flavonoids (Q-quercetin, EGCG-epigallocatechin gallate). The results indicate an expected membrane fluidity increase in more than 50% of the control group for the cells exposed to antioxidants, but a smaller percent increase in the membrane fluidity for the T2DM group, suggesting that administration of only antioxidants cannot restore the normal function of the PBMC in diabetes patients.

We also computed an “apparent binding parameter” following equation (4). Figure 2 clearly shows that there are important statistical differences (p<0.0001) between the apparent binding parameter of TMA-DPH to the cell membrane for the three groups, suggesting a serious impairment in the function of the cell membrane for the T1DM subjects, who are exposed to hyperglycemic stress. Despite that, no significant correlation could be found between the
value of the fasting plasma glucose and the apparent binding parameter, so that another approach has to be taken into account to explain the obtained results.

Incubation with 1 μM quercetin of PBMC isolated from control subjects [14] led to an expected decrease of the fluorescence anisotropy ($r$) with a corresponding increase of the membrane fluidity ($f$) in more than 50% of the subjects (Figure 3), and also did, in a lesser extent, to the T2DM patients. No reliable data could be obtained for T1DM group.

Similar results were obtained following the exposure of PBMC to 10 μM epigallocatechin gallate (Figure 3) [14].

Endothelial dysfunction plays a pivotal role in the initiation and progression of atherosclerosis. In fact, the vascular endothelium modulates the vessel tone by releasing both relaxing and contractile factors, regulates the adherence of mononuclear cells to its surface and the vascular permeability, and produces substances involved in the regulation of hemostasis and tissue proliferation. All these processes have been demonstrated to be altered in human diabetes, which is characterized by a modified adherence of mononuclear cells to the endothelium, an altered vascular permeability, and a hypercoagulable state with platelet activation. These observations have recently led to the hypothesis that a modified interaction between circulating molecules and/or cells and the vessel wall might be at the basis of the endothelial dysfunction in diabetes. The interaction between endothelial cells and circulating blood cells, especially mononuclear cells, might depend on the biophysical properties of the cell membrane [12, 13].

Our results suggest a dysfunction of the cell membrane that can be put into evidence by fluorescence anisotropy measurements with TMA-DPH as a fluorescent reporter. The exposure to flavonoids results in an increase of the membrane fluidity in more than 50% of the subjects belonging to the control group, but in a lesser extent for T2DM patients (27.3% and 44.4% after incubation with 1 μM Q and 10 μM EGCG, respectively), suggesting that the treatment of the cells only with flavonoids cannot suppress the disease-induced changes in the membrane properties.

**Conclusions**

The investigation of the membrane fluidity proved to be a useful and sensitive method to obtain a better insight into the mechanisms by which different compounds, including drugs, can affect the cellular functions.

The study was performed on peripheral blood mononuclear cells (PBMC) separated from diabetes mellitus patients compared to healthy control subjects, using TMA-DPH as fluorescent probe, in steady-state fluorescence polarization experiments.

The T1DM group responded heterogeneously to the molecular reporter, which did not insert into the membrane for most of the patients belonging to this group. Only for 25% of the T1DM patients the $r$ value could be calculated.

The results show that there are no major differences between the baseline membrane fluidity determined for the groups (T1DM, T2DM, control), but a statistically significant difference between the groups in what concerns the ability of the probe to insert into the cell membrane, suggesting possible changes in the membrane properties.

**In vitro** exposure of PBMC to both antioxidant compounds (Q and EGCG) led to an expected increase of the membrane fluidity in more than 50% of the control group, but not for the T2DM group, suggesting that only the antioxidants treatment cannot suppress the changes in the membrane properties, induced by the disease.
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References