Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1768 (2007) 2195–2204
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Modification of membranes by quercetin, a naturally occurring flavonoid, via its incorporation in the polar head group

Bożena Pawlikowska-Pawlęga [a,*], Wiesław Ignacy Gruszecki [b], Lucjan Misiak [b], Roman Paduch [c], Tomasz Piersiak [a], Barbara Zarzyka [d], Jarosław Pawelec [a], Antoni Gawron [a]

[a] Department of Comparative Anatomy and Anthropology, Maria Curie-Sklodowska, University, ul. Akademicka 19, 20-033 Lublin, Poland
[b] Institute of Physics, Maria Curie-Sklodowska University, ul. Akademicka 19, 20-033 Lublin, Poland
[c] Department of Virology and Immunology, Maria Curie-Sklodowska, University, ul. Akademicka 19, 20-033 Lublin, Poland
[d] Department of Cell Biology, Maria Curie-Sklodowska, University, ul. Akademicka 19, 20-033 Lublin, Poland

Received 14 December 2006; received in revised form 28 May 2007; accepted 30 May 2007
Available online 12 June 2007

Abstract

Quercetin is a naturally occurring flavonoid that has a lot of beneficial properties to human health. In this report, using the spin label technique, the influence of quercetin on the fluidity of multilamellar DPPC liposomes was studied. The polarity of the environment preferred by quercetin was also examined by determining the dependence of the position of electronic absorption maxima on dielectric properties of different environments. Autofluorescence of quercetin was also used to examine its distribution in cells. An additional aim of the study was to find how quercetin presence affects human skin fibroblasts. The results showed that incorporation of quercetin at physiological pH into DPPC liposomes caused changes in the partition coefficient of the Tempo spin label between water and polar head group phases. By determining the electronic absorption maxima, we observed that the chromophore of quercetin is localized in the polar head region. Fluorescence microscopy of HSF cells showed quercetin presence in the membrane, cytoplasm and inside the nucleus. Ultrastructural observation revealed some changes, especially in membranous structures, after flavonol treatment. From the results we have concluded that quercetin present in the membrane and other structures can cause changes within cells crucial for its pharmacological activity.

Keywords: Quercetin; DPPC liposome; EPR; Fluorescence microscopy; Electron microscopy

1. Introduction

Flavonoids are a group of naturally occurring, low molecular weight benzo-γ-pyrone derivatives, ubiquitous in plants. Common food of plant origin has from traces to several grams of flavonoids per kilogram of fresh weight [1]. Recently there has been a growing scientific interest in flavonoids, especially in quercetin, due to its potential beneficial properties to human health.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most abundant bioflavonoids commonly present in most edible fruits and vegetables. Quercetin exerts multiple pharmacological effects. It prevents tumor development and carcinogen activation [2]. It arrests the cell cycle [3] and induces apoptosis [3–5]. It is also known that quercetin inhibits heat shock protein expression [6].

By inhibition of $Ca^{2+}Mg^{2+}$ATPase, Na^{+} K^{+} ATPase, mitochondrial ATPase, cAMP- and cGMP-phosphodiesterase, flavonoids affect the membrane ion transport [7,8]. Quercetin is also known to inhibit the enzymatic activity of alkaline phosphatase, phospholipase A_2 and protein kinases [1,9]. Ca^{2+} influx and/or Ca^{2+}metabolism is also influenced by quercetin [10,11]. Quercetin acts as an antioxidant in phospholipid bilayers composed of egg yolk phosphatidylcholine [12]. Thus, within a cell, the membrane seems to be one of the targets for quercetin activity.

Indeed, flavonoids, among them quercetin, and their interactions with the membrane, has been the subject of many articles in recent years. The influence of flavonoids and isoflavonoids on the

* Corresponding author. Fax: +48 81 537 59 01.
E-mail address: pawbo@biotop.umcs.lublin.pl (B. Pawlikowska-Pawlęga).

0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.05.027

fluidity of phosphatidylcholine liposome membranes was investigated by fluorescence polarisation anisotropy measurements [13]. Movileanu et al. examined the effect of quercetin on the electrical properties of the planar lipid bilayers suggesting that the depth of flavonoid embedding is strongly pH-dependent [14]. Hendrich and coworkers using microcalorimetry and absorbance measurements demonstrated isoflavones partitioning into liposomal bilayers [15]. In other articles, in which microcalorimetry technique was also used, quercetin has been demonstrated to partition into DPPC membranes [16,17]. The interaction between flavonoids and membranes of DPPC has also been studied by means of noncovalent immobilized artificial membrane (IAM) chromatography [18].

Areias and coauthors evaluated the degree of membrane partition of the flavonoids. They suggested that the higher antioxidant activity of the flavonoids depends on their capacity to interact and permeate the lipid bilayer [19]. Numerous authors showed correlation between antioxidant activity of flavonoids and their ability to interact and permeate the membrane [19–21].

As it can be seen, flavonoids and their interaction with the membrane are a frequently appearing subject of articles in recent years, but only very few of them apply EPR methods for examination. Hence, considering the widespread occurrence of quercetin in food, its beneficial effects on human health and the data mentioned above, we decided to estimate the action of quercetin upon the membrane. Using the spin label technique, the influence of quercetin on the fluidity of lipid membrane was studied. Physicochemical properties of quercetin were also evaluated. The localization of quercetin within the cell was also examined. We were also interested how quercetin presence affects human skin fibroblasts.

2. Materials and methods

2.1. Chemicals

Quercetin (Sigma Chemical Co., USA) dissolved in ethanol (Merck, Germany) was used in the studies. The solution was kept in the dark. Spin labels: 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical (16-SASL), 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo), 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy free radical (5-SASL), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-diacyl-*sn*-glycero-3-phosphocholine from egg yolk (EYL) were purchased from Sigma Chemical Co. Spin labels were dissolved in absolute ethanol and stored at 4 °C. All other chemicals were of the best quality available.

2.2. Solubility of querectin

Using the following solvents mentioned below: benzene, butanol, n-decane, chloroform, methanol, isopropanol, acetone, glycerol, dietyl ether, and distilled water, saturated solutions of quercetin were prepared. Unilamellar EYL liposomes modified with 5 mol% querectin were prepared as well [22]. EYL was selected because its bilayer phase is fluid at ambient temperature. After short centrifugation (12,000×*g* for 3 min), liposome fractions in the supernatant were collected. Electronic absorption spectra were recorded with Shimadzu 160A—UV-VIS spectrophotometer. On the basis of the spectra, the maxima of light absorption expressed in wave numbers, were determined according to formula $\sqrt{}$ [cm^{-1}]=$10^7/\lambda$ [nm] where λ means a wavelength in nanometers (1). It is known that the position of electronic absorption maxima of chromophores depends on the dialectric properties of their environment [23,24]. Thus, the relationship between the maximum of light absorption and Debye polarisability term: $(n^2-1)/(n^2+2)$ (2), where n stands for a refractive index of an organic solvent applied, was determined. On the basis of such dependence, the polarity of the environment preferred by quercetin can be found.

2.3. Electron paramagnetic resonance (EPR) measurements

Multilamellar liposomes were obtained by shaking. We used saturated DPPC liposomes, which resulted in an ordered phase that was interrupted by an insertion of the flavonoid. Furthermore, in our previous work we examined the effect of quercetin on egg yolk lecithin liposomes representing unsaturated type of membranes. The concentration of the lipid (DPPC) in phosphate buffer was 10^{-5} M. The concentration of quercetin was 5 mol% and of the spin label 1 mol% with respect to the lipid. Dispersion of multilamellar liposomes of DPPC (73 mg/ml of chloroform) was prepared by mixing solutions of respective compounds, evaporation of solvent, first in a stream of nitrogen and subsequently by vacuum (2 h). The samples were hydrated with phosphate buffer (100 mM, pH 8) by vigorous shaking at a temperature above the main phase transition of lipid (>41 °C) until optical homogenity of the mixture was observed. The samples, to be measured, were placed in a 1.3-mm diameter capillary (Hyland Lab. Inc) and sealed with miniseal wax. EPR spectra were recorded with a SE/X-2547 (Radiopan, Poznañ) spectrometer working in the X band and equipped with variable temperature—stabilizing unit under the following conditions: modulation amplitude 5 G in the case of spectra scanning and 10 G for determining an accurate position of the maxima time constant 0.3 s, scan time 2 min, scan range 3200–3300 G. The n-SASL spin labels applied in the study are commonly used to monitor the fluidity of model membranes [22]. In the spectra, the maximum splitting value $2T'_{\|}$, an empirical parameter related to an order parameter of the alkyl chain and the rate of the alkyl motion of the spin label in a lipid core, reflecting the fluidity of a membrane, was analyzed. With the use of the polar spin label Tempo we have also examined the changes of partition coefficient B/A. High-field EPR spectra of Tempo spin label show two peaks: one correspondent to a relatively mobile fraction of spin label in the water phase (A) and the fraction of spin label immobilized within membrane (B) (Fig. 5).

All data are expressed as means±SEM (n=5). Statistical comparison was performed by Student's t-test and values of $p<0.05$ were considered significant.

2.4. Cell culture

HSFs, [human skin fibroblast cell line derived from freshly excised skin fragments in the Department of Virology and Immunology, UMCS, Lublin, Poland] were cultured in RPMI (1640) medium (Roswell Park Memorial Institute) (pH 7.2) supplemented with antibiotics (penicillin—100 j/ml, streptomycin—100 μg/ml, amphotericin B—0.25 μg/ml) and 10% foetal bovine serum (FBS) (v/v). Cells at a density of 1×10^6 were seeded in 7 ml (working volume) tissue culture Falcon vessels for electron microscopy or on the cover slide in Leighton dishes (2 ml) for localization and incubated at 37 °C in humidified 5% CO_2/95% air incubator.

2.5. Localization of quercetin in cells

HSF cells were incubated with quercetin (15 μg/ml) for 30 min, 1 h and for 8 h in the dark at 37 °C. Autofluorescence of quercetin in HSFs on cover glass was analyzed under confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany). At least 100 cells were examined for each experimental variant. The data were registered in the fluorescent channel (λ=458 nm). The relative level of pixel fluorescence was measured along a chosen line passing through the cytoplasm and nucleus using LSM5 Image Examiner software (Zeiss, Germany). As controls, cells were incubated in the absence of quercetin and were not labeled (not shown).

2.6. Transmission electron microscopy

The cells were gently scraped off flasks using a cell scraper. Then they were fixed in 4% glutaraldehyde in 100 mM cacodylate buffer for 2 h and in 1% osmium tetroxide for the next 2 h, all at 4 °C. The cells were dehydrated in series of alcohol and acetone and embedded in LR White resin.

Ultrathin sections were cut with a glass knife on microtome RMC MT – XL (Tucson, AZ, USA), collected on copper grids and contrasted with the use of uranyl acetate and Reynold's liquid. The samples were observed under LEO – Zeiss 912 AB electron microscope (Oberkohen, Germany).

3. Results

3.1. Polarity of the environment preferred by quercetin

Fig. 1 presents the electronic absorption spectrum of quercetin dissolved in glycerol. The position of electronic absorption maxima of chromophores depends on the dielectric properties of their environment [23,24]. Such dependence can be expressed in the form presented in Fig. 2. The figure presents dependence of the absorption maximum expressed in wave numbers (proportional to the energy of the state) on Debye polarisability term: $(n^2-1)/(n^2+2)$ (2), where n is a refractive index of an organic solvent applied. The short wavelength absorption maximum, visible in the region of 250 nm, was selected for the analysis owing to the fact that in all the quercetin solutions examined (in different organic solvents) the band was relatively sharp, which enabled precise localization of the absorption maximum. The two dashed lines in Fig. 2 represent the polarisability term values corresponding to the water phase and to the hydrophobic core of the membrane [25,26]. As it can be seen, the absorption maximum of quercetin incorporated into the membrane, pointed by the arrow marked with letter L, crosses the line that represents the correlation, in the polarisability term region close to the water phase. Such results indicate that the chromophore of quercetin incorporated into the lipid membrane system is localized in the polar head group region rather than in the hydrophobic core of the lipid bilayer.

3.2. The effect of quercetin on structural and dynamic properties of model phosphatidylcholine membranes

To asses the fluidity of membranes, spin labels were introduced to multilamellar DPPC liposomes and examined by EPR technique. Spin labels of n-SASL are commonly used to monitor structural and dynamic properties of lipid bilayer because the shape of their EPR spectra is strongly dependent on the motional freedom of the free radical segment of a spin label molecule within the membrane [22]. The lower the maximum splitting ($2T'_{\|}$, the parameter closely related with the order parameter within the membrane), the higher the fluidity of the membrane in the vicinity of free radicals. Modification of DPPC liposomes with quercetin (5 mol%) had slight (no) effect on the fluidity of the hydrophobic core of the membrane at the depth monitored either by 16-SASL or by 5-SASL. The values of the maximum splitting $2T'_{\|}$ for 16-SASL in the liposomes with quercetin were generally about 0.1 G lower with the exception for the temperature between 17.5 and 35 °C. The differences in the values of $2T'_{\|}$ were then slightly bigger than the sensitivity of the method (±0.66 G) (Fig. 3). The values for 5-SASL were

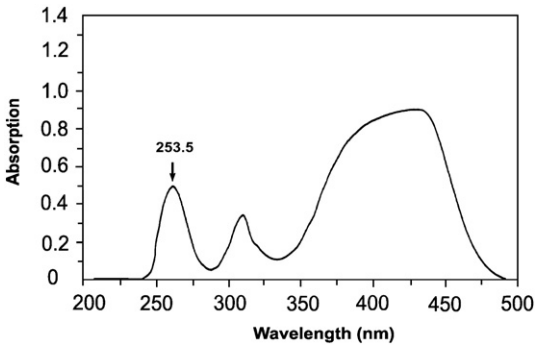

Fig. 1. Electronic absorption spectrum of quercetin dissolved in glycerol. The arrow points the localization of the absorption maximum.

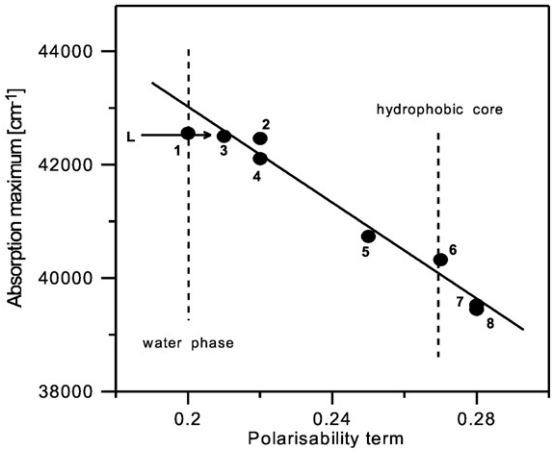

Fig. 2. Dependence of the absorption maximum expressed in wavenumbers (proportional to the energy of the state) on Debye polarisability term. Solvents applied: 1—methanol, 2—acetone, ethanol, 3—dietyl ether, L—phospholipids, 4—isopropanol, 5—decane, 6—chloroform, 7—benzene, 8—glycerol. Dashed lines represent polarisability term values corresponding to the water and hydrophobic core of the membrane. The arrow marked with letter L indicates the absorption maximum of quercetin incorporated to the membrane.

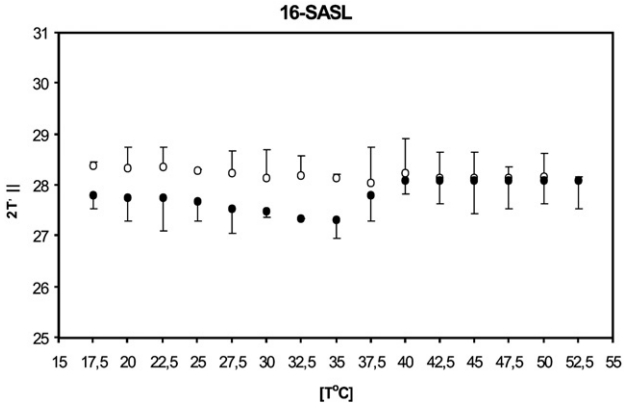

Fig. 3. The effect of quercetin on maximum splitting parameter ($2T'_{\|}$) as a function of temperature of 16-doxylstearic acid (16-SASL) spin label doped into DPPC liposomes. (○) Liposomes of dipalmitoylphosphatidylcholine with addition of spin label at the concentration of 1 mol%. (●) liposomes with quercetin at the concentration of 5 mol% and spin label at the same concentration as mentioned above. The concentration of lipid in 100 mM phosphate buffer was 10^{-5} M. The measurements were performed at various temperatures (17.5–52.5 °C).

0.2–0.3 G lower than in the control (Fig. 4). The pronounced effect of quercetin was observed in the region of polar head groups penetrated by polar spin label Tempo. High-field EPR spectra of Tempo spin label show two peaks: one correspondent to a relatively mobile fraction of spin label in the water phase (A) and the fraction of spin label immobilized within membrane (B) (Fig. 5). In the spectra, the changes of partition coefficient (B/A) were analyzed. The partition coefficient of Tempo spin label between water and lipid phase allows one to determine to which extent the examined compound incorporates into the membrane and to assess membrane fluidity in the polar group region. As it can be seen, at the temperature lower than the temperature of the main phase transition (below 41 °C) quercetin caused the increased penetration of spin label into the polar head groups region. Quercetin addition has changed the ordered structure of lipid membrane into less compact and the spin label probe could easily enter the lipid phase. At the temperature above phase transition (41–47.5 °C), that is, the fluid phase of liposomal membrane where small ordering of lipids occurs, quercetin hindered the penetration of Tempo spin label between the lipids due to its own incorporation. Thus, quercetin decreased partition coefficient (B/A).

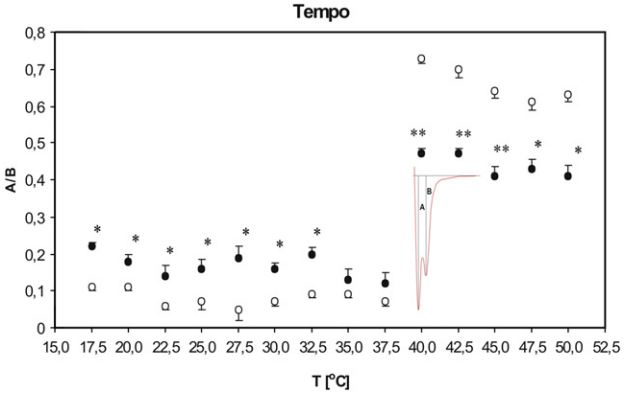

Fig. 5. Temperature dependence of partition coefficient B/A of Tempo spin label between lipid and water phases doped into DPPC liposomes. (○) Liposomes of dipalmitoylphosphatidylcholine with addition of spin label at the concentration of 1 mol%. (●) Liposomes with quercetin at the concentration of 5 mol% and spin label at the same concentration as mentioned above. The concentration of lipid in 100 mM phosphate buffer was 10^{-5} M. A fragment of the high-field EPR spectrum of Tempo spin label doped into DPPC liposomes at temperature 40 °C is added on the right down side. A expresses the intensity of the minimum corresponding to the relatively mobile fraction of the spin label in the water phase and B expresses the shoulder shifted by 2.7 G with respect to the minimum, representing the fraction of the spin label immobilized within the membrane. Each point represents mean ± SEM ($n=5$). *$p<0.05$; **$p<0.01$.

3.3. Localization of quercetin in cells

Quercetin shows autofluorescence that enables its simple detection inside cells. The flavonoid in the concentration of 15 μg/ml was used to examine its distribution in human skin fibroblast cells. Control cells did not exhibit any fluorescence. The short incubation caused fluorescence of regions located around the dark nucleus (Fig. 6A). The relation of nuclear and cytoplasmic fluorescence was 0.28 to 1, respectively (Fig. 7A).

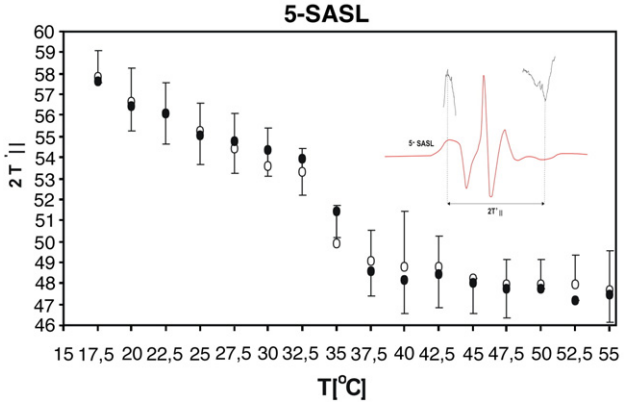

Fig. 4. The effect of quercetin on maximum splitting parameter ($2T'_{||}$) as a function of temperature of 5-doxylstearic acid (5-SASL) spin label doped into DPPC liposomes. (○) Liposomes of dipalmitoylphosphatidylcholine with addition of spin label at the concentration of 1 mol%. (●) Liposomes with quercetin at the concentration of 5 mol% and spin label at the same concentration as mentioned above. The concentration of lipid in 100 mM phosphate buffer was 10^{-5} M. The measurements were performed at various temperatures (17.5–52.5 °C). A typical EPR spectrum of 5-doxylstearic acid spin label incorporated into DPPC liposomes with the measured empirical parameter $2T'_{||}$ is visible on the right up side. The spectrum scanned at modulation amplitude 5 G, maximum at 10 G.

It was found that quercetin was localized within the cell membrane and cytoplasm. Prolonged incubation of HSF cells with quercetin for 1 h slightly increased its fluorescence and was confirmed by a quantitative analysis (Fig. 7B, C). Prolonged treatment with quercetin also changed its distribution. Fluorescence microscopy of HSF cells incubated with the studied drug showed its presence in the cytoplasm, around the nuclear envelope and inside the nucleus and nuclei. 8-h incubation resulted in labeling of the whole cells and revealed a higher level of fluorescence of both cytoplasm and nuclear region (Fig. 6D). The relation of nuclear and cytoplasmic fluorescence was 3.45 to 1 (Fig. 7D).

3.4. Ultrastructure of HSF cells

In order to assess how quercetin presence affects human skin fibroblasts the cells were treated with quercetin at a concentration of 15 μg/ml for 24 h. The cells showed some changes, especially in the membranous structures, after flavonol treatment.

The control cells had typical subcellular organization (Fig. 8A). The mitochondria were uniformly shaped (Fig. 8C). In some cells they represented a condensed form, in others high-energetic ones. The endoplasmic reticulum had well preserved cisternae with ribosomes abutted at the surfaces. The nucleus had homogenous karyoplasms with a well discernible membrane (Fig. 8B).

The cells treated with quercetin were changed. The cell membrane had a lot of protruding processes (Fig. 8G). The cytoplasm was often granular and dark. In the cytoplasm myelin figures were also present (Fig. 8D, E). The endoplasmic reticulum had often widened cisternae. Autofagosomes were also

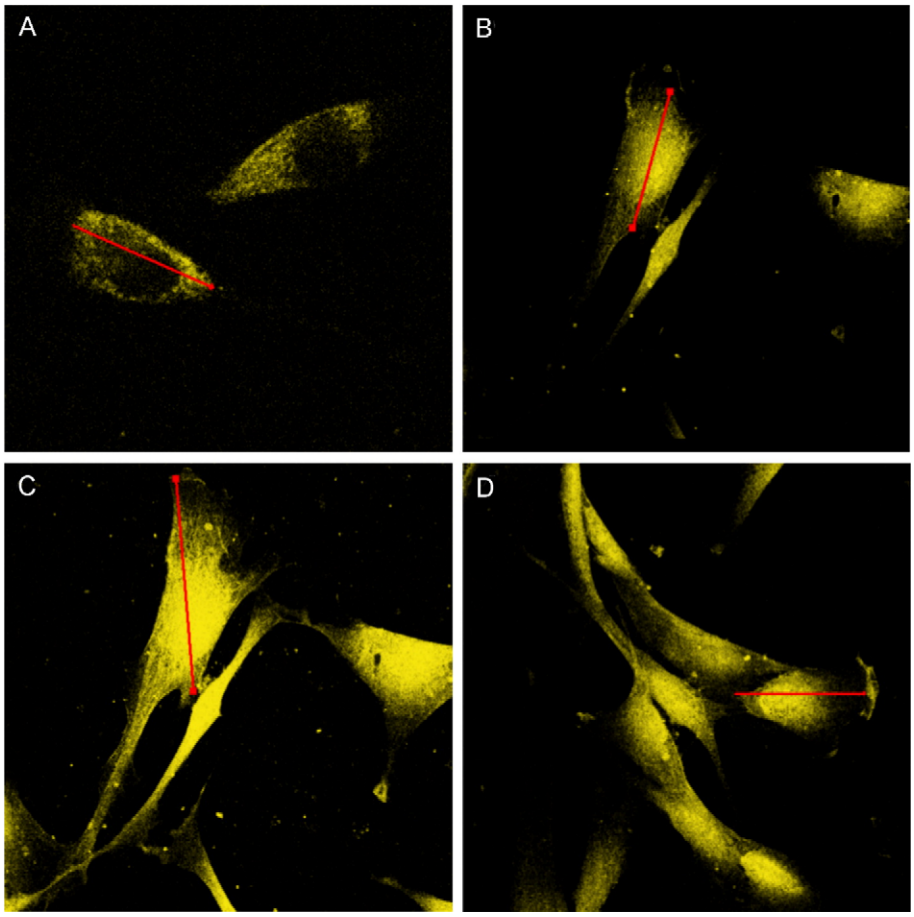

Fig. 6. Distribution of quercetin in HSF cells detected on the basis of its autofluorescence. (A) Cells treated with quercetin for 30 min, (B, C) cells exposed to quercetin for 1 h, (D) cells showing autofluorescence of quercetin after 8 h treatment. Lines visible in the cells are the lines along which the level of fluorescence was measured.

found (Fig. 8E, H). The nuclear envelope showed a locally distorted nuclear membrane. The amount of vacuoles was increased (Fig. 8E, F, H).

4. Discussion

The analysis of absorption spectra of quercetin solutions in different solvents and in liposomes made of egg yolk lecithin revealed high affinity of the chromophore quercetin to low polarity environment including phospholipids. In previous UV-VIS studies on quercetin and other flavonoids it was shown that their spectra exhibit two major absorption peaks in the region 240–400 nm [27]. One of them is commonly referred to as Band I (300–400 nm) and the second as Band II (240–280 nm). Band I is considered to be associated with absorption due to the B-ring cinnamoyl system, and Band II with absorption involving the A-ring benzoyl system. It is important to note that increasing hydroxylation of the A ring causes a notable bathochromic shift in Band II and to a smaller extent on Band I, whereas on increasing the oxygenation of the B-ring, a bathochromic shift in Band I occurs. Our conclusions are based on a well-defined chromophore of the molecule and are in agreement with the results shown in the data obtained by Marby and co-authors. UV spectral data of quercetin also show two peaks in a similar wavelength range.

The chemical structure of quercetin can also predict its affinity to low polarity environment. This flavonoid has an aromatic part which has affinity to lipid component—hydrophobic environment. At the same time the presence of five hydroxyl groups indicates the preferences to polar environment. The amphiphilic character of quercetin molecule enables us to predict its localization in the membrane at the polar–nonpolar interface. Such a localization of quercetin was supported by EPR data [17]. The influence of quercetin on the phospholipid bilayer was indicated by some authors [16,28]. Quercetin decreased peroxidation of lipids isolated from rat brain. It was also suggested that the efficiency of antioxidant activity in the membrane depended on the incorporation rate of the examined compound and on the orientation in biomembrane. Ratty et al., using autofluorescence probe, suggested that flavonoid aglycones interact with the polar group of phospholipids on the surface of the membranes [29]. By using differential scanning calorimetry, Saija et al., demonstrated that quercetin interacts with phospholipid bilayers. The authors suggested that the planar structure of quercetin [28], which was determined by using program Hyperchem ™ Release 2 for Windows (Autodesk, Inc), may explain its high affinity for the membrane. They also claimed that trapping the chain-initiating radicals in the interface of the biomembranes by quercetin makes it an effective protector of progression of the radical chain reaction in

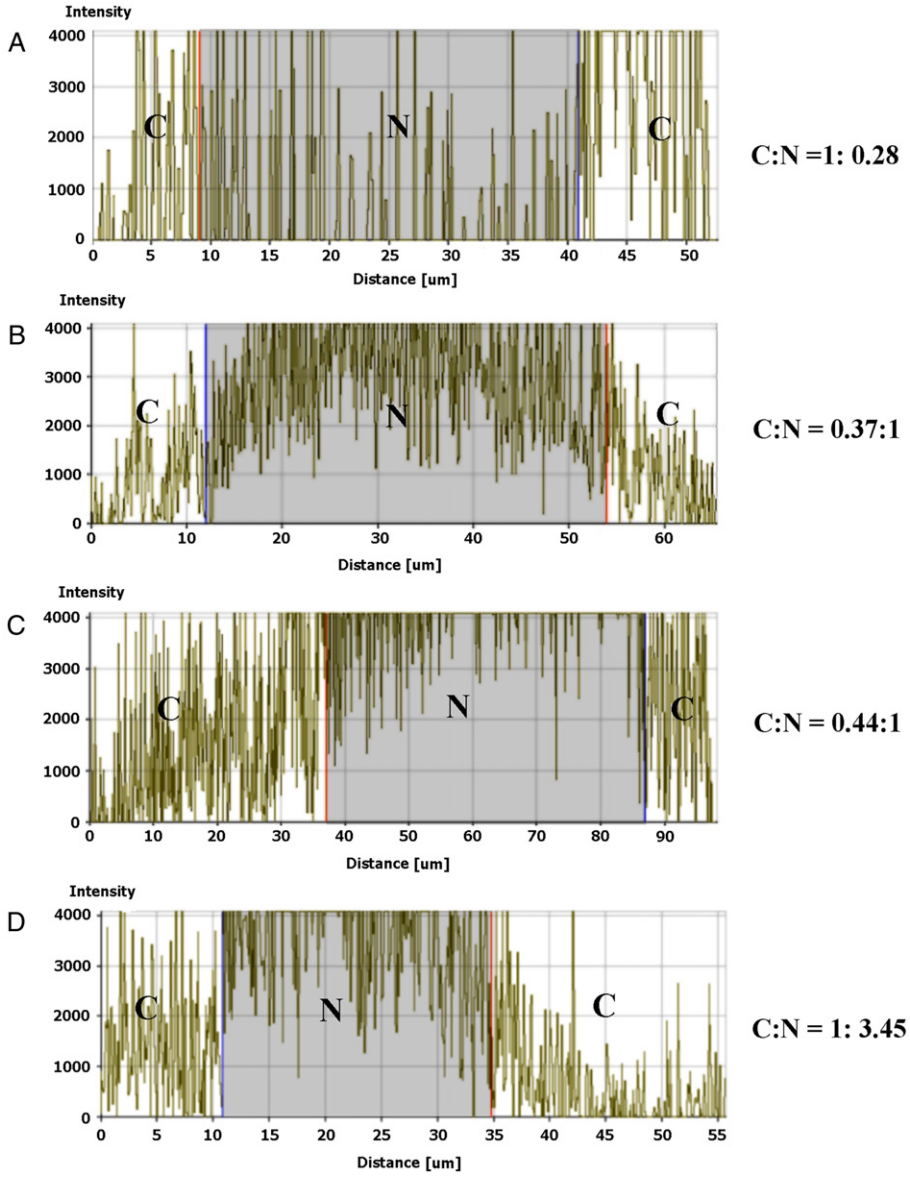

Fig. 7. The level of autofluorescence of quercetin in the nuclear region (N) and cytoplasm (C) of HSF cells. (A) Cells incubated with quercetin at the concentration of 15 μg/ml for 30 min, (B, C) cells treated with quercetin for 1 h, (D) prolonged incubation of HSF cells—lasting 8 h; C:N—relation of the relative cytoplasmic and nuclear fluorescence measured along the chosen line passing through the cell.

membrane lipid peroxidation. In this sense quercetin can be considered as an interfacial antioxidant [16]. Van Dijk and coworkers evaluated the affinity of flavonoids to liposomes by measurements their capacity to quench the fluorescence of a membrane probe. Quercetin showed high affinity and it was attributed to the planar three-dimensional structure of flavonols compared to a tilted configuration of flavanones. At the same time relative hydrophobicity measured as the partition coefficient between water and olive oil, was higher for flavones and flavanones than for flavonols, to which quercetin belongs [30]. The incorporation of the flavonoids in cellular membranes is affected by electrostatic interactions, formation of hydrogen bonds with polar groups of the phospholipids, hydrophobic interaction with fatty acyl chains and by the molecular geometry of phospholipids. Quercetin has a more planar structure due to presence of the double bond at C_2–C_3 on the C ring [19]. Such a structure does not confer high flexibility to conformational changes but rather results in a more rigid structure of quercetin [31].

Localized near the surface of the membrane [17,29], quercetin protects liposomal phospholipids against peroxidation by aqueous oxygen radicals [12] and prevents the consumption of lipophilic α-tocopherol [32]. Quercetin, as it was shown by observation with the use of fluorescent microscope, is present in cells not only in the cell membrane but also in the cytoplasm and nucleus. Due to localization in the structures of similar polarity, it can modify their properties and activities. Final results of such behavior can be the changes in proliferation [33,34], inhibition of enzymatic activity of protein kinases and phospholipase A_2 [1], affecting membrane ion transport [11,8],

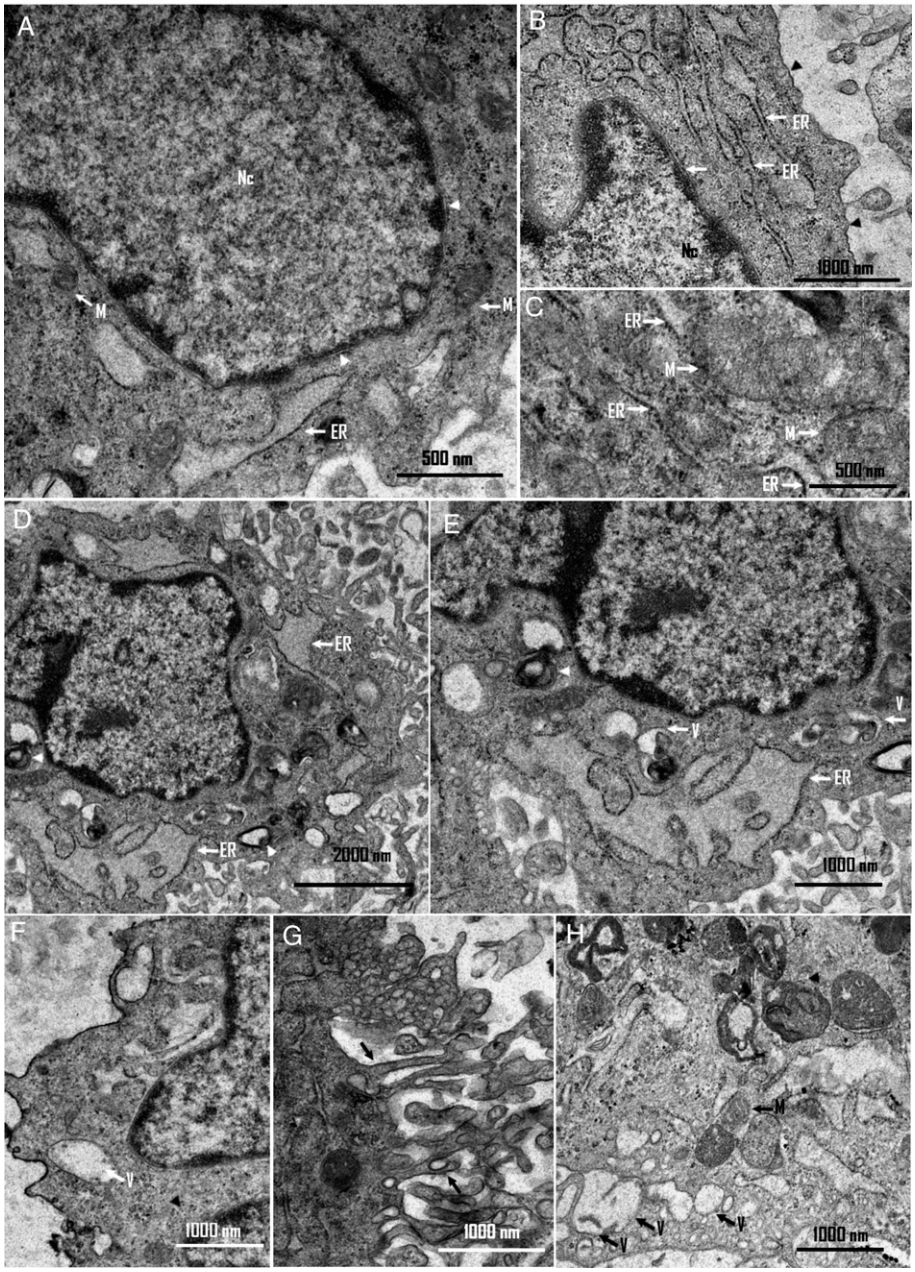

Fig. 8. The ultrastructure of human skin fibroblast cells. Abbreviations: Nc, nucleus; ER, endoplasmic reticulum; M, mitochondria; V, vacuoles. (A) Control cell with a condensed mitochondria (arrows), well-defined endoplasmic reticulum (arrow) and nuclear envelope (arrowhead). In the middle of the nucleus (Nc) homogenous karyoplasm is visible. (B) A portion of control cell with ER (arrows) organized in a typical way, fragment of a "smooth" cell membrane (arrowheads) and a portion of karyoplasm (Nc). (C) A fragment of control cell with uniformly shaped mitochondria (arrows), typically appearing ER with visible ribosomes abutted at the surfaces. (D) A cell treated with quercetin with widened cisternae of ER (arrows), myelin figures (arrowhead). (E) Enlargement view of a fragment of the cell with myelin figures (arrowhead), autofagosomes (V) and widened ER cisternae (arrow). (F) A portion of a cell with visible vacuoles (arrow) and locally distorted nuclear envelope. (G) A fragment of a cell with protrudings of the cell membrane (arrows). (H) A fragment of cytoplasm in quercetin treated cell with visible vacuoles (arrows) and autofagosomes grouped in one place (arrowhead).

decreasing the expression of c-*myc* and ki-ras genes [35] and cell-cycle arrest [3]. Quercetin can also disturb the process of mitosis causing the formation of multinuclear and giant cells [36]. The formation of multinuclear and giant cells was also found by Shimura and co-workers [37]. Additionally, they also found creation of longitudinal cells after quercetin treatment. Such changes may suggest an alteration of cytoskeletal protein. The changes in the shape of human erythrocytes were found in our research [38]. At the same time the observation of localization of quercetin in the cell indicated fluorescence in the regions of cells protrudings. Bound to cytoskeletal elements, quercetin can modify and change the shape of the cell. Our studies on red blood cells with the use of EPR technique indicated quercetin binding to the cytoskeletal proteins [38].

In ultrastructural studies there were visible changes in the endoplasmic reticulum, mitochondria and cell surface mem-

brane. The presence of vacuolation and myelin figures was also noted. As it was shown in examination under fluorescence microscope, quercetin is localized in the perinuclear region, that is, in the place where endoplasmic reticulum is usually abundant. Some cytoplasmic organelles are also present there. It seems that, bound to these elements, quercetin can modify their structure and their activities.

In the present study the effect of quercetin on structural and dynamic properties of the membrane was observed with the use of multilamellar liposomes made of DPPC. The spin-labels used provided information about the membrane interior (in the vicinity of the spin label). The shape of EPR spectra strongly depends on the motional freedom of the free radical segment of the spin label molecule within the membrane [22]. We used 16-SASL which enabled the examination of the membrane in the hydrophobic core of the lipid membrane. 5-SASL tested the order of acyl chains in the hydrophobic region adjacent to the polar heads. The tempo spin label penetrated the membrane in the region of the polar head groups. In the spectra, the maximum splitting parameter ($2T'_{\|}$, the parameter closely related with the order parameter within the membrane) and partition coefficient (B/A) of Tempo spin label into the membrane were analyzed. The lower the maximum splitting, the higher the fluidity of the membrane in the vicinity of free radicals. The partition coefficient between water and lipid phase enables determination to which extent the compounds examined are incorporated into the membrane and, at the same time, assessment of the fluidity (order) in the region of polar head groups. High field spectra of Tempo spin label show two peaks: one correspondent to a relatively mobile fraction of the spin label in the water phase (A) and the fraction of the spin label immobilized within membranes (B) (Fig. 5).

The analysis of the partition coefficient value of Tempo spin label showed an increased penetration of the spin label into the membrane at the temperature below the temperature of the phase transition (solid state of lipids). The addition of quercetin changed the ordered structure of lipid membrane to less compact (higher fluidity) and the molecule of the spin label could more easily penetrate into the lipid phase. Above the temperature of the phase transition, quercetin behaved in different way. In a liquid state of liposomal membrane, i.e., in the condition of bigger freedom (mobility of lipids), quercetin made the spin label penetration more difficult due to its own incorporation into membrane and its spatial localization. That is why the partition coefficient lowered. The activity of quercetin in the polar head region of lipid bilayer of DPPC was shown by Ratty and coworkers [29]. Quercetin present in the bilayer is able to scavenge free radicals and inhibit the activity of lipoxygenase-1 in liposomal suspension by about 42%. Such behavior of quercetin was explained not only by the chemical structure of quercetin but also by the ability of penetration and interaction with biomembranes [16]. Localized at the polar–nonpolar interface, it causes to some extent disturbances of the normal order of lipid matrix. Quercetin can be also fully integrated into the membrane. It was shown on the planar lipid bilayer that quercetin insertion is strongly pH-dependent [14]. The deepest insertion occurs in acidic medium. In such conditions quercetin is neutral and completely liposoluble. In contrast, within alkaline media deprotonation of quercetin happens and the reaction site of the flavonoid is restricted to the hydrophilic domain of the membrane. Negatively charged quercetin molecules become sandwiched between polar headgroups at the bilayer surface. The protonation degree was also discussed in the work of others [18]. They claim that it has a marked effect on flavonoids partitioning between the polar and nonpolar phase of DPPC membranes. They showed that an increase in the pH of the water phase favors the hydrophilic character due to deprotonation of the hydroxyl groups. They also suggested that flavonoids with more hydroxyl groups stronger interact with DPPC membrane interface. Oteiza and co-authors also indicated binding of more hydrophilic flavonoids at the membrane surface [20,21,39]. Translocation of quercetin in the polar part of the lipid bilayer, at physiological pH, may be correlated with its protective ability against different deleterious agents including radical scavenging activity [18,21]. The data are also consistent with Terao and co-workers results for liposomes [32].

We applied pH 8 in our experiment. Thus, the finding from the current investigation agrees well with those of others [14], who found that at such pH no insertion of quercetin into the hydrophobic core of the membrane was observed but an intercalation of quercetin between the polar head groups of phospholipids took place. This is also in good agreement with the work of Ioku and co-authors, who suggested that at physiological pH quercetin interacts with the polar head group phospholipids by locating itself at the membrane surface [12]. As it was shown in this paper, quercetin is localized in the polar head group region of phospholipids. Quercetin present in the watery phase affects the acyl chains conformation. It is known that as a result of isomeric transformation of "gauche-trans" type such a bending of acyl chains may result, which may even cause possible contact between quercetin and molecules at distant parts of acyl chains of phospholipids. The final effects of such interactions are the changes in lipid chain arrangement and an increase in the membrane fluidity. Additionally, the effects may also include the increase in ultrasound absorption during the change of the phase, as it was indicated in our earlier experiments [17]. In our previous work on DPPC membranes [17] we also observed a decrease in cooperativity and temperature of the phase transition of liposomes. The shift of the phase transition temperature toward lower values combined with the broadening effect of temperature profiles. These results were similar to the effects recorded for quercetin—DPPC membranes in the work of others [16]. Similarly, for flavonoids other than those studied in the present work, supporting results were found. The authors suggested that the decrease in the lipid gel–liquid crystalline transition temperature (T_m) and the transition enthalpy (ΔH) taken together with broadening of the transition peaks indicate flavonoid penetration into the lipid membrane and presumable location near the polar–nonpolar interface of the lipid leaflet [15]. Using the spin label located at different depths of the lipid bilayer, we supported that quercetin is located in the polar group region at physiological pH. It was mentioned above that the depth of quercetin embedding strongly depends on pH

[14]. At acidic conditions quercetin particles penetrate into the hydrophobic region of the bilayer. At physiological pH its location is shifted further towards the aqueous environment [14,32,38].

In the papers mentioned above [16,17], a decrease in T_m in dipalmitoylphosphatidylcholine liposomes indicated a less ordered membrane. On the contrary, an increase in membrane lipid packing was observed for flavonoids and isoflavonoids (there was no querectin there) in phosphatidylcholine liposome membranes [31]. These opposite findings may be connected with the various compositions of the membranes under study. In the first one, the usage of dipalmitoylphosphatidylcholine liposomes resulted in an ordered phase that was interrupted by the incorporation of the flavonoids. In the second study, Arora and co-workers applied the unsaturated 1-stearoyl-2-linoleoyl phosphatidylcholine liposomes, which resulted in a less ordered phase. Hence, the insertion of the flavonoid in the bilayer core determined a higher ordering of the acyl chains, thus increasing the T_m of the membrane.

Thus, quercetin causes to some extent the disturbances of normal order of lipid matrix. The molecules of a compound cause a disorder which is especially pronounced in a well-organized (ordered) liquid-crystallic phase of DPPC membrane. These changes indicate that "impurity effect" might have occurred [40].

In conclusion, the present study shows a modifying effect of quercetin on phospholipids membranes. Localized in the polar head group region at physiological pH, quercetin changes the properties of the cell membrane. Its effects on lipids, especially fluidization of membrane, are very important for membrane functioning. They may induce or inhibit membrane-dependent regulation mechanisms. These may be followed by the changes within the cells being crucial for pharmacological activity of quercetin used in cancer or other disease treatment.

Acknowledgement

This work was funded by the Maria Curie-Sklodowska University in Lublin.

References

[1] J.V. Formica, W. Regelson, Review of the biology of quercetin and related bioflavonoids, Food Chem. Toxicol. 33 (1995) 1061–1080.
[2] M. Richter, R. Ebermann, B. Marian, Quercetin-induced apoptosis in colorectal tumor cells: possible role of EGF receptor signaling, Nutr. Cancer 34 (1999) 88–99.
[3] Y. Wei, X. Zhao, Y. Kariya, H. Fukala, K. Teshigawara, A. Uchida, Induction of apoptosis by quercetin: involvement of heat shock protein, Cancer Res. 54 (1994) 4952–4957.
[4] B. Pawlikowska-Pawlęga, J. Jakubowicz-Gil, J. Rzymowska, A. Gawron, The effect of quercetin on apoptosis and necrosis induction in human colon adenocarcinoma cell line LS 180, Folia Histochem. Cytobiol. 39 (2001) 217–218.
[5] J. Rzymowska, A. Gawron, B. Pawlikowska-Pawlęga, J. Jakubowicz–Gil, J. Wojcierowski, The effect of quercetin on induction of apoptosis, Folia Histochem. Cytobiol. 37 (1999) 125–126.
[6] N. Hosokava, K. Hirayoshi, A. Nakai, Y. Hosokava, I. Marui, M. Yoshida, T. Sakai, H. Nishino, A. Aoike, K. Kawai, K. Nagata, Flavonoids inhibit the expression of heat shock proteins, Cell Struct. Funct. 15 (1990) 393–401.
[7] Y. Graziani, R. Chayoth, Regulation of cyclic AMP level and synthesis of DNA, RNA and protein by quercetin in Ehrlich ascites tumor cells, Biochem. Pharmacol. 28 (1979) 397–403.
[8] M.P. Revuelta, B. Cantabrana, A. Hidalgo, Depolarization-dependent effect of flavonoids in rat uterine smooth muscle contraction elicited by CaCl2, Gen. Pharmacol. 29 (1997) 847–857.
[9] M. Lindahl, Ch. Tagesson, Flavonoids as phospholipase A2 inhibitors; importance of their structure for selective inhibition of group II of phospholipase A2, Inflammation 21 (1997) 347–354.
[10] M.A. Morales, X. Lozoya, Calcium-antagonist effects of quercetin on aortic smooth muscle, Planta Med. 60 (1994) 313–317.
[11] B. Pawlikowska-Pawlęga, K. Trębacz, E. Król, A. Gawron, Effects of quercetin and verapamil on membrane potential in the liverwort *Conocephalum conicum*, Acta Physiol. Plant. 1 (2000) 61–68.
[12] K. Ioku, T. Tsuhida, Y. Tokei, I. Nakatani, Antioxidative activity of quercetin and quercetin monoglucosides in solution and phospholipid bilayers, Biochim. Biophys. Acta 1234 (1995) 99–104.
[13] A. Arora, T. Byrem, M. Nair, G. Strasburg, Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids, Arch. Biochem. Biophys. 373 (2000) 102–109.
[14] L. Movileanu, I. Neagoe, M.L. Flonta, Interaction of the antioxidant flavonoid quercetin with planar lipid bilayers, Int. J. Pharm. 205 (2000) 135–146.
[15] A.B. Hendrich, R. Malon, A. Pola, Y. Shirataki, N. Motohashi, K. Michalak, Differential interaction of Sophora isoflavonoids with lipid bilayers, Eur. J. Pharm. Sci. 16 (2002) 201–208.
[16] A. Saija, M. Scalese, M. Lanza, D. Marzullo, F. Bonina, F. Castelli, Flavonoids as antioxidant agents: importance of their interaction with biomembranes, Free Radical Biol. Med. 19 (1995) 481–486.
[17] K. Wójtowicz, B. Pawlikowska-Pawlęga, A. Gawron, L.E. Misiak, W.I. Gruszecki, Modifying effect of quercetin on the lipid membrane, Folia Histochem. Cytobiol. 34 (1996) 48–50.
[18] F. Ollila, K. Halling, P. Vuorela, H. Vuorela, J.P. Slotte, Characterization of flavonoid–biomembrane interactions, Arch. Biochem. Biophys. 399 (2002) 103–108.
[19] F.M. Areias, A.C. Rego, C.R. Oliveiria, R.M. Seabra, Antioxidant effect of flavonoids after ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells, Biochem. Pharmacol. 62 (2001) 111–118.
[20] P.I. Oteiza, A.G. Erlejman, S.V. Verstraeten, C.L. Keen, C.G. Fraga, Flavonoid–membrane interactions: a protective role of flavonoids at the membrane surface? Clin. Dev. Immunol. 12 (2005) 19–25.
[21] A. Lopez-Revuelta, J.I. Sanchez-Gallego, A. Hernandez-Hernandez, J. Sanchez-Yagüe, M. Llanillo, Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress, Chem. Biol. Interact. 161 (2006) 79–91.
[22] W.K. Subczyński, I. Markowska, W.I. Gruszecki, J. Sielewiesiuk, Effect of polar carotenoids on dimirystylophosphatidylocholine membranes: a spin label study, Biochim. Biophys. Acta 1105 (1992) 97–108.
[23] P.O. Andersson, T. Gilbro, L. Fergusson, Absorption spectral shifts of carotenoids related to medium polarizability, Photochem. Photobiol. 54 (1991) 353–360.
[24] A. Sujak, J. Gabrielska, J. Milanowska, P. Mazurek, K. Strzałka, W.I. Gruszecki, Studies on canthaxanthin in lipid membranes, Biochim. Biophys. Acta 1712 (2005) 17–28.
[25] R.J. Cherry, D. Chapman, Refractive index determination of lecithin black films, J. Mol. Biol. 30 (1967) 551–553.
[26] M. Gagos, R. Koper, W.I. Gruszecki, Spectrophotometric analysis of organisation of dipalmitoylphosphatidylcholine bilayers containing the polyene antibiotic amphotericin B, Biochim. Biophys. Acta 1511 (2001) 90–98.
[27] T.J. Marby, K.R. Markham, M.B. Thomas, The Systematic Identification of Flavonoids, Springer-Verlag, New York, 1970.
[28] J. Terao, M. Piskula, Flavonoids and membrane lipid peroxidation inhibition, Nutrition 15 (1999) 790–791.
[29] A.K. Ratty, J. Sunamoto, N.P. Das, Interaction of flavonoids with 1,1-diphenyl-2picrylhydrazyl free radical, liposomal membranes and soybean lipoxygenase, J. Biochem. Pharmacol. 6 (1988) 989–995.

[30] C. van Dijk, A. Driessen, K. Recourt, The uncoupling efficiency and affinity of flavonoids for vesicles, Biochem. Pharmacol. 60 (2000) 1593–1600.
[31] A. Arora, M. Nair, G. Strasburg, Antioxidant activities of flavonoids and their biological metabolites in a liposomal system, Arch. Biochem. Biophys. 356 (1998) 133–141.
[32] J. Terao, M. Piskula, O. Yao, Protective effect of epicatechin, epicatechin gallate and quercetin on lipid peroxidation in phospholipid bilayers, Arch. Biochem. Biophys. 308 (1994) 278–284.
[33] N. Hosokava, Y. Hosokava, T. Sakai, M. Yoshida, N. Marui, N. Nishino, K. Kawai, A. Aoike, Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle related 17 kDa protein in human colon cancer cells, Int. J. Cancer 45 (1990) 1119–1124.
[34] M. Yoshida, T. Sakai, N. Hosokava, N. Marui, K. Matsumoto, A. Fujioka, N. Nishino, A. Aoike, The effect of quercetin on cell cycle progression and growth of human gastric cancer cells, FEBS Lett. 260 (1990) 10–13.
[35] B. Csokay, N. Prajda, G. Weber, E. Olah, Molecular mechanism in the antiproliferative action of quercetin, Life Sci. 60 (1997) 2157–2163.
[36] B. Pawlikowska-Pawlęga, A. Gawron, Effect of quercetin on the growth of mouse fibroblast cells in vitro, Pol. J. Pharmacol. 47 (1995) 531–535.
[37] M. Shimura, Y. Zhou, T. Yoshikawa, K. Hatake, F. Takaku, Y. Ishizaka, Inhibition of Vpr-induced cell cycle abnormality by quercetin: a novel strategy for searching compounds targeting Vpr, Biochem. Biophys. Res. Commun. 261 (1999) 308–316.
[38] B. Pawlikowska-Pawlęga, W.I. Gruszecki, L.E. Misiak, A. Gawron, The study of the quercetin action on human erythrocyte membranes, Biochem. Pharmacol. 66 (2003) 605–612.
[39] A. Scheidt, A. Pampel, L. Nissler, R. Gebhardt, D. Huster, Investigation of the membrane localization and distribution of flavonoids by high-resolution magic angle spinning NMR spectroscopy, Biochim. Biophys. Acta 1663 (2004) 97–107.
[40] K. Strzałka, W.I. Gruszecki, Effect of b-carotene on structural and dynamic properties of model phosphatidylcholine membranes. An EPR spin label study, Biochim. Biophys. Acta 1194 (1994) 138–142.