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Research Paper

Time-dependent effect of rutin on skin fibroblasts membrane disruption following UV radiation

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ABSTRACT

Chronic exposure of the skin to solar UV radiation induces a number of biological alterations, including a redox imbalance; therefore, there is an urgent need for skin cells protective compounds. The aim of this study was to determine the effects of natural, previously extensively examined, polyphenol with antioxidant properties – rutin, on UV-induced skin fibroblasts membrane disruption. Accordingly, fibroblasts exposed to UVA and UVB irradiation were incubated with rutin (12 h before and/or up to 24 h after irradiation), and the structural and metabolic changes were examined. Rutin penetration through the fibroblast phospholipid bilayer was aided by UVA-induced bilitranslocase activity 2–4 h after irradiation, while UVB irradiation led to enhanced phospholipid peroxidation and higher membrane permeability to facilitate the interaction of rutin with phospholipids. Lipidomic analysis revealed that 4 h of rutin treatment also partially prevented UVA/B-induced increase in phosphatidylethanolamine and phosphatidylcholine level, as well as their membrane localization, which resulted in an enhanced zeta potential in the cells and liposomes. Moreover, rutin 2 h following irradiation, in a various degree, prevented the increased in phospholipase A2 activity and ROS generation, and partially protected against the reduction of arachidonic and linoleic acids level and the lipid peroxidation product 4-hydroxynonenal level increase. Rutin effectively prevented against decrease in glutathione peroxidase, glutathione and vitamins E and C activities/levels, particularly 2 h following UVA irradiation.

In conclusion, highest skin fibroblasts membrane level of rutin occurred in 2–4 h following UVA/B-radiation results in its strongest effect on biomembrane structure and functions and cellular antioxidant system irrespective of the radiation type.

1. Introduction

Over the last decade, studies on various skin disorders related to increased solar ultraviolet radiation have grown continuously. Chronic exposure of mammalian skin to UV radiation induces a number of biological alterations that are directly or indirectly involved in the development of skin cancer and photoaging [1,2].

UV radiation is a potent initiator of photochemical reactions via the excitation of electrons that can result in energy transfer and/or chemical modifications of exposed molecules. UV radiation reaching the Earth's surface consists of UVA and UVB rays. UVA radiation penetrates deeply through the epidermis into the dermis and is involved in the generation of ROS, including singlet oxygen, hydrogen peroxide and hydroxyl radicals, and is therefore considered to be the aging ray. UVB radiation mostly affects the epidermal basal cell layer of the skin and also induces direct and indirect adverse biological events in epidermal and dermal cells [3]. A key and essential property of all cells, including skin cells, is their ability to maintain homeostasis,

Abbreviations: 4-HNE, (4-hydroxynonenal); ABTS, (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CAT, (catalase); CE, (capillary electrophoresis); CMH, (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetrame-thylpyrrolidine); COX, (cyclooxygenase); cPLA$_2$, (cytosolic phospholipase A$_2$); DAD, (diode array detector); DMSO, (dimethyl sulfoxide); DTNB, (5,5-dithio-bis-(2-nitrobenzoic acid)); ESI, (electrospray ionization); ESR, (electron spin resonance); FAME, (fatty acid methyl ester); FID, (flame ionization detector); GC, (gas chromatography); GSH, (glutathione); GSH-Px, (glutathione peroxidase); GSSG, (glutathione disulfide); GSSG-R, (glutathione reductase); GST, (glutathione S-transferase); HILIC, (hydrophilic interaction liquid chromatography); HPLC, (high-performance liquid chromatography); HRP, (horseradish peroxidase); LDH, (lactate dehydrogenase); LOX, (lipoxygenase); MS, (mass spectrometry); *O*-PFB-oxime-TMS, (*O*-(2,3,4,5,6-pentafluoro-benzyl)-oxime-trimethylsilyl); PBS, (phosphate-buffered saline); PC, (phosphatidylcholine); PE, (phosphatidylethanolamine); PI, (phosphatidylinositol); PLs, (phospholipids); PS, (phosphatidylserine); PUFA, (polyunsaturated fatty acid); QTOF-MS, (quadrupole time-of-flight mass spectrometry); ROS, (reactive oxygen species); SM, (sphingomyelin); SOD, (superoxide dismutase); TFA, (trifluoroacetic acid)

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especially redox balance. Cells are in a stable state when the rate of ROS generation is balanced by the action of enzymatic and nonenzymatic antioxidants that permit ROS-mediated intracellular signaling and redox regulation of genes [4]. However, an elevated ROS level that cannot be counteracted by the endogenous antioxidant system induces a redox imbalance, resulting in oxidative stress. This situation provokes oxidative modifications of the structures and functions of cellular components and consequently damages skin cells.

Among the various cellular components, the most sensitive to oxidative modifications by ROS and other electrophiles are membrane phospholipids, which contain high levels of polyunsaturated fatty acids (PUFAs) [5]. The most powerful initiators of lipid peroxidation are hydroxyl and hydroperoxyl radicals. The former are generated from hydrogen peroxide during cellular oxygen metabolism via the Fenton and Haber–Weiss reactions in the presence of free iron or copper ions and as a result of high-energy irradiation [6]. Moreover, UV radiation that enhances the activity of NADPH and xanthine oxidases in keratinocytes and fibroblasts leads to the generation of a superoxide anion that, in its protonated form (a hydroperoxyl radical), is a sufficiently strong oxidant that can initiate the oxidation of PUFAs and transform hydrogen peroxide to a hydroxyl radical [7,8]. As a result, ROS-dependent peroxidation leads to the formation of metabolically active products of lipid cyclization and fragmentation, which is enhanced in fibroblasts and keratinocytes 24 h after UVA and UVB irradiation [8]. The lipid peroxidation products are longer-lived than ROS and initiate chain reactions that further enhance oxidative damage; this is believed to be responsible for the development of skin cancer due to DNA damage [9]. However, ROS are constantly generated in skin cells; keratinocytes and fibroblasts are also rapidly removed by the antioxidant system. This prevents the harmful effects of ROS and maintains the redox balance, thus resulting in cell and tissue stabilization. Although skin cells possess an efficient antioxidant system, extensive or chronic exposure to UV radiation may exceed their antioxidant abilities, leading to oxidative disturbances [10].

Therefore, to protect the skin from the harmful effects of UV radiation, it is necessary to identify a natural photoprotective compound. Skin care products are enriched in vitamins C, E, A and β-carotene, but increasing attention has recently been paid to the antioxidant and photoprotective properties of polyphenolic compounds that can be used as nutrients or for topical application [11]. The large group of polyphenols that possess antioxidant properties are flavonoids, which include rutin (a quercetin glycoside) [12,13]. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid that is found in buckwheat, apple, green tea, betula leaves and other sources [14]. It has been clearly proven that rutin possesses antioxidant and anti-inflammatory activities [15]. Its antioxidant properties are dependent on the presence of an *o*-dihydroxy group in the B-ring (a catechol structure), which is the most important potential radical target [16]. However, the C-5 and C-7 hydroxyl groups (of the A ring) are potential free radical scavengers, particularly if they are combined with a catechol B-ring structure. However, a double bond between the C-2 and C-3 of the C-ring conjugated with the keto group in position 4 (because of its capacity to delocalize the uncoupled electron of the flavonoid radical) also contributes to antioxidant activity [17]. The presence of phenolic hydroxyl groups and π-electron conjugation in the flavonoid structure favors the scavenging and inactivation of free radicals, which is correlated with their capacity to inhibit phospholipid bilayer destruction [18]. The long-term effects of rutin protection on redox balance in fibroblasts exposed to UV irradiation are well-known [19]; however, the abilities of rutin to penetrate the biomembrane and interact with its components under physiological and oxidative stress conditions, particularly immediately after irradiation, are not fully understood. The mechanism of the penetration of natural polyphenols, e.g., flavonoids, in membranes remains controversial. Because rutin partially eliminates the negative effect of UV irradiation on fibroblasts, it is important to establish the rutin-UV radiation interaction mechanism with respect to the fibroblast membrane.

Therefore, the aim of this study was to compare the effects of UVA and UVB radiation on rutin penetration through the cellular membrane of fibroblasts cultured as a monolayer when rutin was applied before and after UV irradiation. The effectiveness of rutin treatment and pre-treatment on structural and functional changes in the biomembrane was examined in the context of fibroblast redox balance.

2. Methodology

2.1. Cell culture and treatment

Human fibroblasts (CCD 1112Sk) obtained from the American Type Culture Collection were cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) contained fetal bovine serum (10%) and supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. When the cells (passage 8) reached 70% confluence, they were exposed to UV radiation. The cells were irradiated from the 6 lamps (Bio-Link Crosslinker BLX 312/365; Vilber Lourmat, Germany) assembly 6 W each, which corresponds to 4.2 mW/cm^2 and 4.08 mW/cm^2, respectively for UVA (365 nm) and UVB (312 nm). Totalled radiation doses were 20 J/cm^2 and 200 mJ/cm^2 for UVA and UVB respectively. UV radiation doses were chosen corresponding to 70% cell viability, while the concentration of rutin (25 μM) used in the experiment was selected according to 95% cell viability measured by the MTT assay [20] (data not shown).

To determine the effects of rutin on UVA/UVB-irradiated fibroblasts, the following groups were examined:

a/ Cells cultured in medium containing 0.2% DMSO→control.

b/ Cells incubated in medium containing 25 μM rutin (in 0.2% DMSO solution)→rutin.

c/ UVA/UVB-irradiated cells and after irradiation cultured in medium containing 0.2% DMSO→UVA or UVB.

d/ Cells irradiated with UVA/UVB rays, and treated with rutin after irradiation by cultured in medium containing 25 μM rutin (in 0.2% DMSO solution)→UVA + R or UVB + R.

e/ Cells pre-treated with rutin (for 12 h), and irradiated with UVA/UVB and after irradiation cultured in medium containing 25 μM rutin (in 0.2% DMSO solution)→R + UVA + R or R + UVB + R.

After incubation for 1, 2, 4, 6, 12 or 24 h, all cells were washed with PBS; collected by scraping into cold PBS; and centrifuged. Cells were then resuspended in PBS and sonicated. The total protein content in the cell lysate was measured using a Bradford assay [21] and the results were normalized to milligrams of protein.

2.2. Rutin translocation into fibroblasts

2.2.1. Rutin level in the cytosol and phospholipid membrane

Cells were resuspended in 20 mM Tris-HCl buffer, sonicated and centrifuged (15000*g*, 10 min) to separate the cytosolic fraction. The obtained pellet was dissolved in a buffer containing surfactants (50 mM Tris-HCl, 1% Triton X-100% and 0.1% SDS). The suspension was sonicated and centrifuged (15000*g*, 10 min) to obtain the membrane fraction.

The level of rutin in the cytosol and membrane was measured using an Agilent 1290 HPLC system according to method described by Jacobs [22]. The mobile phase consisted of water containing 0.1% (v/v) TFA with linear gradients of 5% acetonitrile at t = 0 to 20% acetonitrile at 5 min, followed by an increase to 30% acetonitrile at 10 min. Finally, 90% acetonitrile was used from 18 min onward for 5 min. The column was re-equilibrated with 5% acetonitrile for 5 min. A flow rate of 2 ml/min and injection volume of 20 μl were used. Detection was carried out on a diode array detector (DAD). The presented data are based on detection at 360 nm (the absorption maximum of rutin).

2.2.2. Bilitranslocase activity

The bilitranslocase transport activity was assessed on a spectrophotometer as described in detail by Passamonti [23]. Sample containing 2 μg proteins in 10 mM Hepes, pH 7.4, 0.25 M sucrose, and 0.15 M NaCl was added to the cuvette containing 1 ml 0.1 M potassium phosphate, pH 8.0 and 45 μM sulfobromophthalein. To initiate K^+ diffusion 5 μg of valinomycin in methanol was added. The decrease in absorbance at the wavelength 580 nm is called electrogenic sulfobromophthalein uptake and 1 μmol of uptaken sulfobromophthalein was defined as 1 unit of bilitranslocase transport activity. The results were normalized to protein concentration and expressed as units per milligram of protein.

2.3. Changes in fibroblast cellular membrane properties

2.3.1. Phospholipid membrane composition

To lipidomic analysis cells were detached using trypsin and the cell suspension was immediately diluted with PBS (1:1) and centrifuged 10 min at 2000 rpm. The cell pellet was washed three times with 1 ml PBS, followed by centrifugation at 2000 rpm for 4 min, and cells resuspended in 1 ml H_2O. Total lipids from all cell lines were extracted with the Bligh and Dyer method [24] descripted in previous paper [25].

Phospholipid composition of fibroblasts was analyzed by ultraperformance liquid chromatography couple to high resolution mass spectrometer (QTOF 6540 Agilent, Santa Clara, CA). Analysis of PLs was carried out in positive and negative. PL classes were separated by hydrophilic interaction liquid chromatography (HILIC). The characterization of individual phospholipid species within each class was achieved by data dependent ESI–QTOF–MS/MS. Chromatographic and electrospray mass spectrometry conditions have been previously described in detail [25]. HILIC-MS/MS analysis of fibroblasts confirmed the presence of phosphatidylcholine (PC), phosphatidylethanolamines (PE), as most abundant classes, as well as presence of sphingomyelins (SM), phosphatidylinositol (PI) and phosphatidylserines (PS). Sphingomyelin classes were also identified.

2.3.2. Phospholipid and free fatty acids membrane composition

Fatty acids profile of fibroblast cells phospholipids was determined by gas chromatography [26]. Lipids components were isolated by Folch extraction using chloroform/methanol mixture (2:1, v/v) in the presence of 0.01% butylated hydroxytoluene. Using TLC free fatty acids and total phospholipids were separated with the mobile phase: heptane – diisopropyl ether – acetic acid (60:40:3, v/v/v). All lipid fractions were transmetylated to fatty acid methyl esters (FAMEs) with boron trifluoride in methanol reagent under nitrogen atmosphere without previous separation from the layer material at 100 °C for 30 min for phospholipids and at 100 °C for 5 min for free fatty acids. FAMEs were analyzed by gas chromatography with a flame ionization detector (FID) on Clarus 500 Gas Chromatograph (Perkin Elmer). Separation of FAMEs was carried out on capillary column coated with Varian CP-Sil88 stationary phase (50 m x 0.25 mm, ID 0.2 μm, Varian). Operating conditions were as follows: the split-splitless injector was used in split mode with a split ratio of 1:20. The injection volume of the sample was 2 μl. The injector and FID detector temperatures were kept at 260 °C. The column temperature was programmed from 150 °C (2 min) to 230 °C (10 min) at 4 °C/min. Identification of FAMEs was made by comparison on their retention time with those authentic standards (methyl linoleate (18:2(n-6)), methyl arachidonate (20:4(n-6)) and methyl nonadecanoate (19:0)). Quantitation was achieved using an internal standard method (nonadecanoic acid (19:0) and 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine (19:0 PC) were used as internal standards). Details of analysis were described previously [8]. The phospholipid and free fatty acid concentrations were expressed as a microgram per milligram protein.

2.3.3. Phospholipase A2 activity

Cytosolic Phospholipase A_2 ($cPLA_2$) activity was measured using $cPLA_2$ Assay Kit (Cayman, No. 765021) according to kit instructions. To detect $cPLA_2$ activity arachidonoyl thio-PC as a substrate was used. Hydrolysis of the arachidonoyl thioester bond at the sn-2 position by PLA_2 releases a free thiol which can be detected by DTNB [27]. Enzyme specific activity was calculated in nanomol of free thiol released per minute per milligram of protein.

2.3.4. Phospholipid peroxidation

Lipid peroxidation was estimated by measuring the level of free 4-hydroxynonenal (4-HNE). Aldehyde was measured by GC-MS/MS, as the *O*-PFB-oxime-TMS derivative, using modified method of Luo et al. [28]. Benzaldehyde-D_6 was added as an internal standard to the cell lysates and aldehydes were derivatized by the addition of *O*-(2,3,4,5,6-pentafluoro-benzyl) hydroxylamine hydrochloride (0.05 M in PIPES buffer, 200 μl) and incubating for 60 min at room temperature. After incubation, samples were deproteinized by the addition of 1 ml of methanol and *O*-PFB-oxime aldehyde derivatives were extracted by the addition of 2 ml of hexane. The top hexane layer was transferred into borosilicate tubes, and evaporated under a stream of argon gas followed by the addition of *N,O*-bis(trimethylsilyl) trifluoroacetamide in 1% trimethylchlorosilane. Derivatized aldehydes were analyzed using a 7890A GC – 7000 quadrupole MS/MS (Agilent Technologies, Palo Alto, CA) equipped with a HP-5 ms capillary column (0.25 mm internal diameter, 0.25 μm film thickness, 30 m length). Derivatized aldehydes were detected in selected ion monitoring (SIM) mode. The ions used for 4-HNE-PFB-TMS identification were: *m/z* 333.0 and 181.0. The 4-HNE concentration was expressed as a nanomol per milligram protein.

2.3.5. Membrane integrity

Cellular membrane integrity was monitored using the assay based on the determination of the release of LDH into the medium. The activity of LDH in medium, as well as in cell lysates was measured as described by Fotakis et al. [20]. The final concentrations of NADH and pyruvate in sample were 1 mM and 2 mM respectively. Detection was carried out at 340 nm on a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA). The percentage release of LDH from the treated cells was calculated by comparing it activity in medium to cell lysates.

2.3.6. Zeta potential measurements

In order to determine zeta potential of cell membrane, fibroblasts or liposomes were suspended in 0.9% NaCl and put into the measuring vessel, then zeta potential was measured using Zetasizer Nano ZS apparatus (Malvern Instruments).

2.3.7. Preparation of liposomes

Liposome dispersions were prepared by the sonication of asolectin (a mixture of phospholipids) from soybean (Fluka). Dry lipids were weighed and dissolved in chloroform (10 mg/ml). The solvent was evaporated under a gentle stream of argon to obtain a dry lipid film. The film was then hydrated with 0.9% NaCl. Liposomes were formed by sonicating the suspension using an ultrasound generator UD 20 (Techpan, Poland). Sonication was applied five times for 1.5 min each. Since heat is liberated during the process; therefore, cooling the suspension is necessary. Cooling was performed in an ice bath (a container with a mixture of ice and dry NaCl).

2.4. Effect of rutin on fibroblasts redox balance

2.4.1. Determination of ROS generation

The generation of reactive oxygen species in cells was detected using an electron spin resonance (ESR) spectrometer e-scan (Noxygen GmbH/Bruker Biospin GmbH, Germany), where selective interaction of the spin probes CMH (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetrame-

thylpyrrolidine, 200 μM) with ROS formed a stable nitroxide CM-radical with a half-life of 4 h [29]. Thus, ROS formation was measured from the kinetics of nitroxide accumulation according to the electron spin resonance amplitude of the low field component of ESR spectra. The generation of ROS was described in micromoles of ROS per minute per milligram of protein. Details of analysis were described previously [8].

2.4.2. Glutathione peroxidase activity

Glutathione peroxidase (GSH-Px – EC.1.11.1.6) activity was assessed spectro-photometrically by measuring the conversion of NADPH to $NADP^+$ using the method of Paglia and Valentine [30]. One unit of GSH-Px activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH min^{-1} at 25 °C and pH 7.4. Enzyme specific activity was calculated in milliunits per milligram of protein and expressed as a percentage of the enzyme specific activity determined from the control cells. Details of analysis were described previously [8].

2.4.3. Glutathione concentration

The levels of two forms of glutathione were quantified using the capillary electrophoresis (CE) method of Maeso [31]. Details of analysis were described previously [8]. The cell lysates were sonicated in Eppendorf tubes with 2 ml of a mixture containing AcN/H_2O (62.5:37.5, v/v) and centrifuged at 30000*g* for 10 min. The supernatant was immediately measured by CE. Separation was performed on a 47 cm capillary (40 cm effective length) and 50 m i.d. and was operated at 27 kV with UV detection at 200 ± 10 nm. The GSH and GSSG concentrations were determined using a calibration curve range of 1–120 nmol/L ($r^2 - 0.9985$) and normalized for milligrams of protein. The results ware expressed as a ratio GSH/GSSG.

2.4.4. Determination of vitamins E and C

HPLC methods were used to detect the levels of vitamins E [32] and C [33]. Details of analysis were described previously [8]. Cell lysates were first centrifuged at 1000 × *g* for 10 min. Vitamin E was extracted from the cell lysates using hexane. The hexane phase was removed, and the remaining mixture was dried and diluted in ethanol before 50 μl of it was injected onto the RP-18 column. UV detection at 294 nm was applied. The flow rate was 1 ml/min of methanol and water (95:5). For determination of vitamin C supernatants were mixed with an equal volume of metaphosphoric acid. Separation was performed using RP-18 column and UV detection at 250 nm. The mobile phase was phosphate buffer (pH = 2.8) and water (97:3). The flow rate was 0.7 ml/min. The concentrations of vitamin E and C were determined using a calibration curves range of 5–25 mg/L and 1.25–20 μg/ml for vitamin E and vitamin C, respectively. The results were normalized for milligrams of protein. The correlation coefficients of the curves for vitamin E and C were $r^2 = 0.9999$ and $r^2 = 0.9998$, respectively.

2.5. Oxidative-antioxidative properties in the presence of rutin in vitro

2.5.1. Scavenging of superoxide radicals

The superoxide radical scavenging activity of rutin was determined according to Den Hartog [34]. Superoxide radicals were generated by xanthine 0.1 mM and xanthine oxidase 10 mU/ml. To detect superoxide radical scavenging mixture was incubated with differ rutin concentration [25–200 μM] for 1 h. The radicals generation was detected using an electron spin resonance (ESR) spectrometer e-scan (Noxygen GmbH/Bruker Biospin GmbH, Germany) [29]. ROS formation was measured from the kinetics of nitroxide accumulation according to the electron spin resonance amplitude of the low field component of ESR spectra. The generation of superoxide anions was described in superoxide anion micromolar concentration per minute and the radical scavenging activity of rutin was expressed as a ratio of superoxide anion generation in control sample to sample incubated with rutin.

2.5.2. Scavenging of hydroxyl radicals

The hydroxyl radical scavenging activity of rutin was determined according to Haenen [35]. The hydroxyl radicals are formed in the reaction between Fe^{2+} and H_2O_2. The reaction mixtures contained rutin [25–200 μM], deoxyribose [2.8 mM], ascorbic acid [100 μM], H_2O_2 [2.8 mM], and $FeCl_3$ in a phosphate buffer [20 mM, pH = 7.4] was incubated 1 h at 37 °C. To stop the reaction 2% TCA was added, and mixture was heated at 100 °C for 20 min. Samples were cooled to room temperature and absorbance was measured at 532 nm. The competition between rutin and deoxyribose for a reaction with hydroxyl radicals was calculated as a ratio of control sample absorbance to sample absorbance with rutin.

2.5.3. Total antioxidant capacity

Total rutin antioxidant capacity was measured in it reaction with $ABTS^{\cdot+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical). ABTS radical solution (12.3 mg/ml) in 50 mM phosphate buffer pH 7.4 was prepared by heating (70 °C) for 20 min 50 μl of 5–200 μM rutin solution was added to 950 μl of the ABTS radical solution and decrease in absorption at 734 nm was measured immediately. Analyses were performed in three times repeat. The total antioxidant capacity of rutin was expressed as a decrease in absorption per minute.

2.5.4. Effects of GSH on rutin oxidation

To determine the influence of GSH (40 μM) on rutin (50 μM) oxidation, the following solutions were prepared: rutin, oxidized rutin, GSH, oxidized GSH, a mixture of rutin and GSH, and an oxidized mixture of rutin and GSH. Samples were oxidized by 1.6 nM HRP and 33 μM H_2O_2 in 145 mM phosphate buffer (pH 7.4) at 37 °C. The levels of rutin and GSH following the reaction were determined by HPLC or CE as previously described.

To examine rutin oxidation in time spectrophotometric analysis was performed at 360 nm on a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA). The detection scans began 50, 150 and 300 s after the addition of HRP and H_2O_2.

2.6. Statistical analysis

All analyses on cells were performed on the results obtained from three independent experiments. The remaining experimental results are presented as the mean of five independent experiments. Data were analyzed using standard statistical methods, including one-way Student's *t*-test for multiple comparisons, and the results are expressed as the mean ± standard deviation (SD). P-values less than 0.05 were considered significant.

3. Results

The experiment was performed on control cells, UVA- and UVB-irradiated cells, rutin-treated cells, and rutin pre- and/or post-UV exposure treated cells. However, due to a lack of statistically significant differences between the 6, 12 and 24 h incubations after UV irradiation, the results from the 12 and 24 h incubations were omitted in the following descriptions. Consequently, the figures primarily show results obtained only at 1, 2, 4 and 6 h following UV exposure. Rutin solutions were simultaneously prepared in 0.2% DMSO, which was omitted in control cells. However, this concentration of DMSO is sufficiently low, and previous experiments have shown that it does not result in any statistically significant change in the analyzed parameters.

3.1. Rutin translocation into fibroblasts

Both UVA and UVB radiation significantly influenced rutin translocation from the medium into fibroblasts (Fig. 1). In both cases, between 2 and 4 h following irradiation, higher levels of rutin were observed in

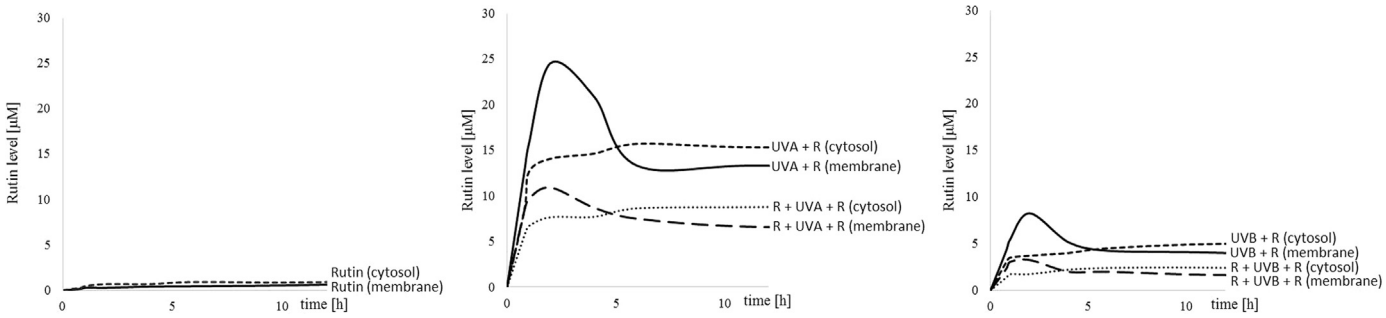

Fig. 1. Changes in the rutin level in fibroblasts exposed to UVA [20 J/cm^2] and UVB [200 mJ/cm^2] radiation and following rutin [25 μM] treatment and pre-treatment over time. Mean values of three independent experiments are presented.

the membrane fractions and decreased thereafter. However, the rutin level in the cytosol increased during the first hour and was maintained after further incubation. Despite exhibiting a similar pattern of action, UVA led to a 2.5-fold increase in the rutin levels in both the membrane and cytosolic fractions relative to UVB radiation. Simultaneously, cells pre-treated before UVA and UVB radiation showed a 2-fold decrease in rutin levels in both fractions compared to cells incubated with rutin only after irradiation (Fig. 1).

The changes in the rutin level in the cytosol following UV irradiation were concomitantly observed with the increase in the activity of the only known specific rutin transmembrane transporter, bilitranslocase. UVA radiation immediately following the 2 h incubation with rutin caused an approximate 70% increase in bilitranslocase activity compared to control cells, while pre-treatment with rutin increased enzyme activity by only approximately 30%. Additionally, a longer incubation with rutin caused an approximate 10% decrease between 2 and 6 h and in the case of rutin pre-treatment and a continued increase in bilitranslocase activity, as observed in fibroblasts without rutin pre-treatment. A much lower dependence on bilitranslocase was observed following UVB irradiation, as shown by approximately 30% and 25% increases after a 2 h incubation following radiation in cells treated with rutin only after exposure and for rutin pre-treated cells, respectively. Incubations longer than 2 h following UVB irradiation did not significantly alter bilitranslocase activity in these cells in either case (Fig. 2).

3.2. Changes in the fibroblast cellular membrane structure and properties

Our lipidomic approach revealed that the phospholipid pattern in fibroblasts is typical of that observed for eukaryotic membranes (Fig. 3). Supplementation with rutin prevents UV-induced changes in the structure and properties of the fibroblast membrane. The fibroblast membrane phospholipid profile was significantly altered after 4 h following UVA and UVB irradiation compared to control cells. An increase in the relative amount of phosphatidylethanolamine and phosphatidylcholine was observed, while the relative amount of

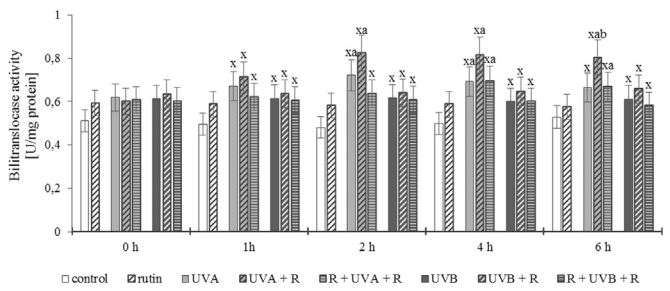

Fig. 2. Time-dependent changes in bilitranslocase activity in fibroblasts following UVA [20 J/cm^2] and UVB [200 mJ/cm^2] radiation and rutin [25 μM] treatment and pre-treatment. Mean values ± SD of three independent experiments are presented. ^{x} statistically significant differences vs. control group, $p < 0.05$; ^{a} statistically significant differences vs. rutin treated group, $p < 0.05$; ^{b} statistically significant differences vs. UV irradiated group, $p < 0.05$.

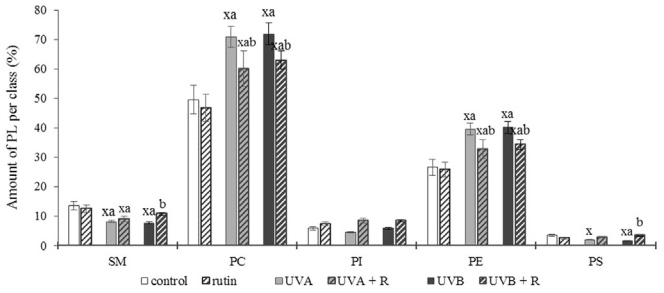

Fig. 3. Changes in the fibroblast membrane phospholipid levels after a 4 h incubation following UVA [20 J/cm^2] and UVB [200 mJ/cm^2] irradiation and rutin [25 μM] treatment and pre-treatment. Mean values ± SD of three independent experiments are presented. ^{x} statistically significant differences vs. control group, $p < 0.05$; ^{a} statistically significant differences vs. rutin treated group, $p < 0.05$; ^{b} statistically significant differences vs. UV irradiated group, $p < 0.05$.

sphingomyelin was significantly decreased after exposure to UV irradiation. UV radiation led to a decrease in the relative abundance of SM species, namely, SM(d18:1/16:0), SM(d18:0/16:0), SM(d18:0/18:0) and SM(d18:1/22:1) (data not shown). However, the amount of phosphatidylinositol (PI) and phosphatidylserine (PS) did not differ significantly between the experimental groups. Fibroblasts incubated with rutin following UV irradiation did not exhibit the observed changes in PC and PE amounts (Fig. 3). The lack of statistical significance between cells treated with rutin pre- and/or post-UV exposure caused these results to be omitted.

Changes in the amounts of particular fibroblast phospholipid classes after UV irradiation are associated with changes in the levels of some polyunsaturated fatty acids (PUFAs), including arachidonic and linoleic acid, which are phospholipids. The level of linoleic acid is decreased by UVB (up to 50% after 1 h), while UVA radiation decreases linoleic acid by up to 20% (after 4 h). Rutin treatment after a 4 h incubation prevented these reductions by nearly half in the case of UVA and by one-third in UVB-radiated cells. Moreover, rutin pre-treatment resulted in a greater protective potential in the early hours than post-treatment. However, after a 4 h incubation, the levels of linoleic acid in pre- and post-treated cells were equal. In the case of arachidonic acid, UVA radiation more effectively decreased its level by up to 30% after 2 h, while UVB radiation decreased the level of arachidonic acid to 35% after 6 h. Rutin supplementation prevented the time-dependent reduction in this phospholipid by as much as 50% compared to irradiated cells (Fig. 4A and B).

A decrease in the levels of phospholipid fatty acids is associated with enhanced activation of PLA2, the main enzyme responsible for releasing fatty acids from the second carbon group of glycerol, which was the highest 2 h after UVA (by 100%) and UVB (by 70%) irradiation. Rutin treatment following UVA irradiation caused a decrease in PLA2 activity (by 15% and 40% post- and pre-treatment, respectively). Conversely, rutin prevented changes caused by UVB radiation by 25% and 30% in cells treated only after UVB radiation and in cells pre-

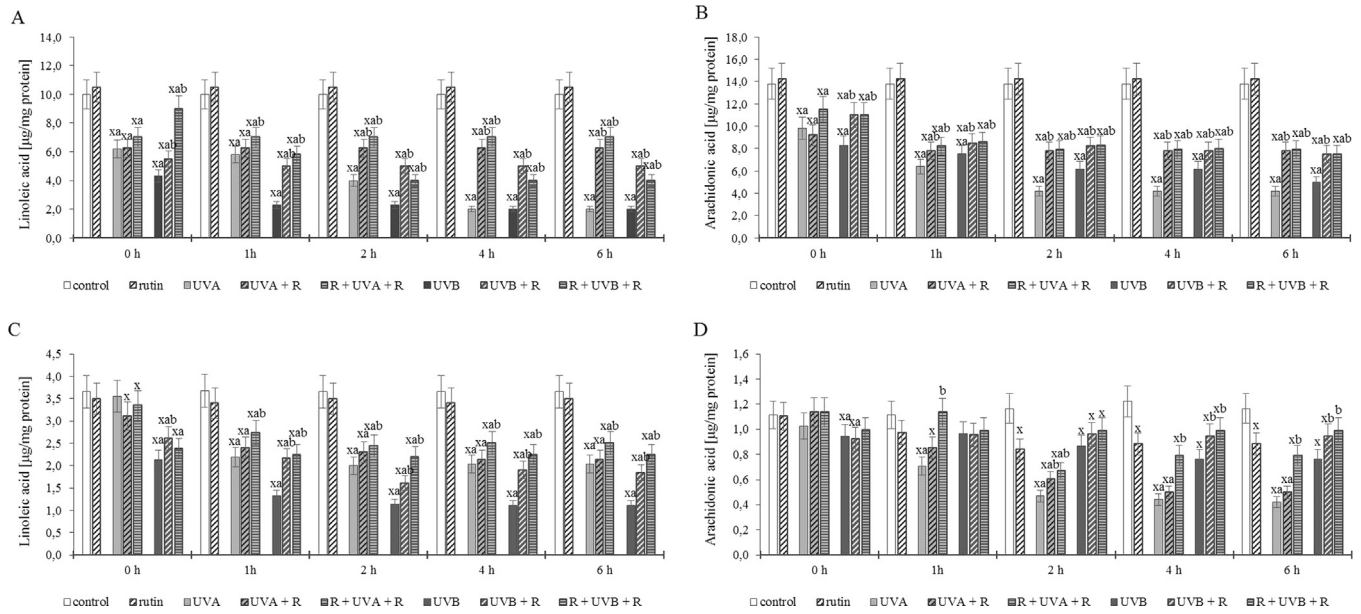

Fig. 4. Time-dependent changes in the level of phospholipid (A-B) and free (C-D) fatty acids (linoleic and arachidonic acid) in fibroblasts following UVA [20 J/cm^2] and UVB [200 mJ/cm^2] radiation and rutin [25 µM] treatment and pre-treatment. Mean values ± SD of three independent experiments are presented. [x] statistically significant differences vs. control group, $p < 0.05$; [a] statistically significant differences vs. rutin treated group, $p < 0.05$; [b] statistically significant differences vs. UV irradiated group, $p < 0.05$.

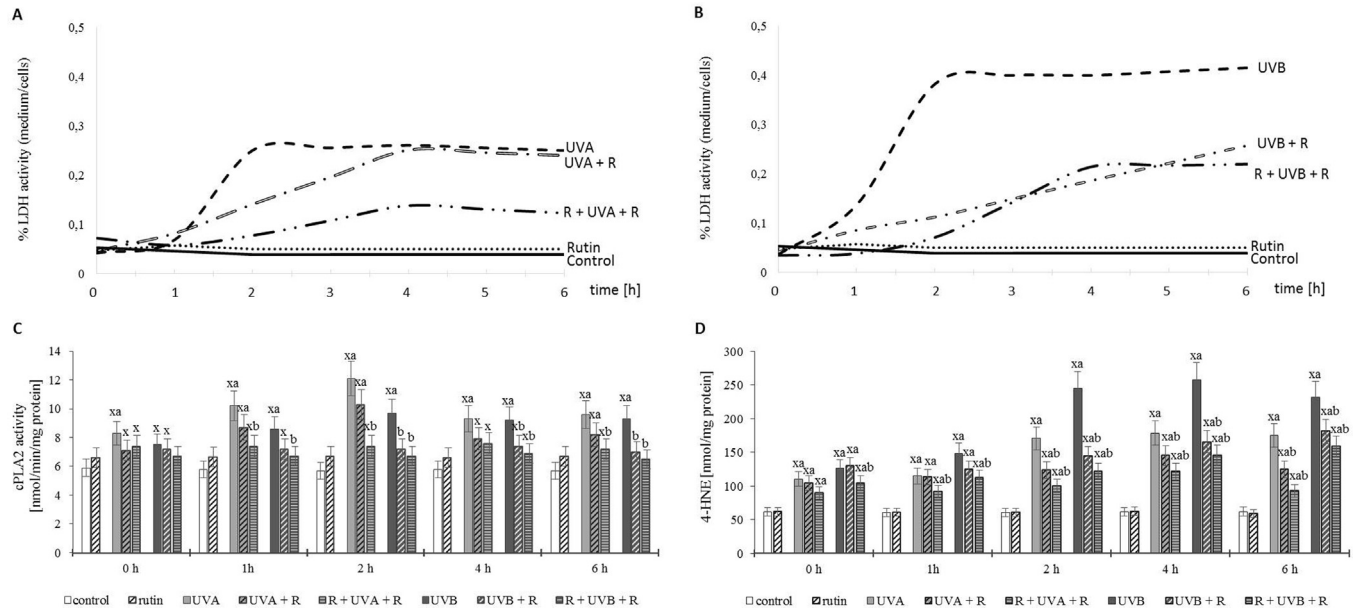

Fig. 5. Time-dependent changes in membrane structure following UVA [20 J/cm^2] and UVB [200 mJ/cm^2] radiation and rutin [25 µM] treatment and pre-treatment, expressed as % of LDH activity released into the medium (A-B), as well as cytosolic phospholipase A2 activity (C) and the level of the lipid peroxidation product 4-HNE (D). Mean values ± SD of three independent experiments are presented. [x] statistically significant differences vs. control group, $p < 0.05$; [a] statistically significant differences vs. rutin treated group, $p < 0.05$; [b] statistically significant differences vs. UV irradiated group, $p < 0.05$.

treated with rutin, respectively (Fig. 5C).

Independent of the decrease in fibroblast phospholipid PUFAs, UV irradiation also decreased the free PUFA level. After 4 h, UVA caused the highest decrease in arachidonic acid, approximately 60%, while UVB only led to a 30% decrease in arachidonic acid. However, the response of linoleic acid to UVB was a stronger decrease after 2 h (approximately 60%) compared to UVA (approximately 40%). Moreover, pre- and post-treatment with rutin strongly (by approximately 50% compared to irradiated cells) protected cells against the UVB-induced decrease of the linoleic acid level in the free fatty acid fraction. By contrast, rutin caused a time-dependent decrease in the free arachidonic acid level of 40% compared to control cells after 2–6 h of incubation. However, rutin pre-treatment stronger than post-treatment prevented decreases in the free arachidonic acid level following UVA radiation, a 45% higher level of this acid after 4 h and 6 h of incubation compared to UVA irradiated cells (Fig. 4C and D).

Changes in the membrane phospholipid composition observed after rutin treatment of UV-irradiated fibroblasts resulted in modifications of the zeta potential. Rutin significantly decreased the zeta potential of cells exposed to UVB radiation, which was mostly observed 1 and 2 h after irradiation and, in the case of UVA radiation, following 2–6 h (Fig. 6). Rutin was also embedded in the liposome membrane structure, thus reducing its negative zeta potential. Moreover, UVA and UVB radiation facilitated this incorporation after only 2 h of incubation with rutin. Under the influence of UVA and UVB, liposomes exhibited decreased negative zeta potential values compared to untreated lipo-

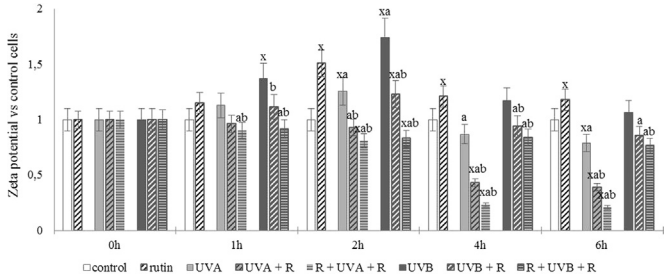

Fig. 6. Time-dependent changes in the zeta potential in fibroblasts following UVA [20 J/cm^2] and UVB [200 mJ/cm^2] radiation and rutin [25 μM] treatment and pre-treatment. Mean values ± SD of three independent experiments are presented. ^{x} statistically significant differences vs. control group, $p < 0.05$; ^{a} statistically significant differences vs. rutin treated group, $p < 0.05$; ^{b} statistically significant differences vs. UV irradiated group, $p < 0.05$.

somes (Fig. 7), corresponding with a decrease in negatively charged phospholipids of fibroblasts following UV irradiation.

Through a reduction in the fatty acid levels, rutin prevents phospholipid peroxidation and biomembrane destruction; this was apparent in the 4-HNE level, which significantly increased immediately and 1 h following UVA and UVB radiation (approximately 2.5-fold for both types of radiation) and continued to increase after this time (to 3.5- and 6-fold for UVA and UVB, respectively). The level of 4-HNE decreased significantly by rutin pre- and post- treatment 2 h following irradiation (Fig. 5D).

Rutin protects cells against disruptions in the membrane composition as well as a breach in membrane integrity. The percent of LDH activity in the medium compared to its cellular activity was 6- and 8-fold higher 2 h following UVA and UVB irradiation, respectively, in membrane permeability compared to control and rutin-treated cells. In the case of cells exposed to UVA radiation, rutin supplementation after exposure delayed the onset of such damage from 2 to 4 h, while rutin pre-treatment delayed and reduced the damage by 2-fold compared to UVA-irradiated cells. Moreover, rutin treatment following UVB radiation, as well as rutin pre-treatment, delayed the onset of such damage from 2 to 4 h and reduced the damage by 2-fold compared to UVB-irradiated cells (Fig. 5A and B).

3.3. Effects of rutin on the fibroblast redox balance

Rutin partially protects cells against a UV-induced redox imbalance. UVA and UVB radiation caused approximately 2- and 2.5-fold increases in ROS generation after a 2 h incubation compared to control cells. Rutin treatment decreased the UV-induced changes by approximately 15% in both cases; however, rutin pre-treatment strongly counteracted the effects of UVA as well as UVB radiation within the first 2 h by approximately 10% compared to cells treated with rutin only after UV radiation (Fig. 8A and B). Additionally, the GSH to GSSG ratio was maximally decreased 2 h following UVA and UVB radiation (approximately 50% of that observed in control cells) and continued to decrease over the next several hours. Rutin post-treatment as well as pre-treatment after a 4 h incubation further prevented UVA- and UVB-induced reductions of the GSH/GSSG ratio (Fig. 8E). Moreover, pre- and post-UVB-irradiated cells treated with rutin did not show decreased activity of GSH-Px, which is responsible for peroxide reduction, after incubation for 4 or 6 h. In the case of UVA-induced reduction in GSH-Px activity, the protective effect was only observed in pre-treated cells (Fig. 8C and D).

Rutin concomitantly prevented UVA- and UVB-induced decreases in the levels of other antioxidants (vitamins E and C). Both UVA and UVB radiation caused a time-dependent decrease in vitamin E and C levels (between 0 and 6 h of incubation to approximately 40% for vitamin E, and 70% (UVA) and 60% (UVB) for vitamin C compared to control cells). Rutin partially prevented these changes and maintained the level of vitamin E at 60–70% of the control. Rutin treatment simultaneously decreased the level of vitamin C following UVB radiation to that observed after a 2 h incubation. However, in fibroblasts pre-treated with rutin, the vitamin C level was maintained at 90% of control cells (Fig. 8F and G).

3.4. Oxidative-antioxidative properties in the presence of rutin in vitro

Rutin may prevent intracellular oxidative stress due to its antioxidant properties in vitro, as shown in Fig. 9. At low concentrations, rutin exhibits a strong ability to reduce ABST (a three-fold increase in the range of 10–30 μM). This scavenging activity is more effective in the case of superoxide radicals than hydroxyl radicals. Rutin (75 μM) reduced the superoxide radical level by 50%, but reduced hydroxyl radicals by only 30%. To reduce the level of hydroxyl radicals by 50%, approximately 150 μM rutin is required.

Moreover, under oxidative conditions, the decrease in rutin level occurs approximately 10% more rapidly in the control solution than in the presence of GSH (Fig. 10A). Following oxidation, the level of rutin decreased by approximately 70%, while in the presence of GSH, the rutin level was 20% of the control value (Fig. 10B).

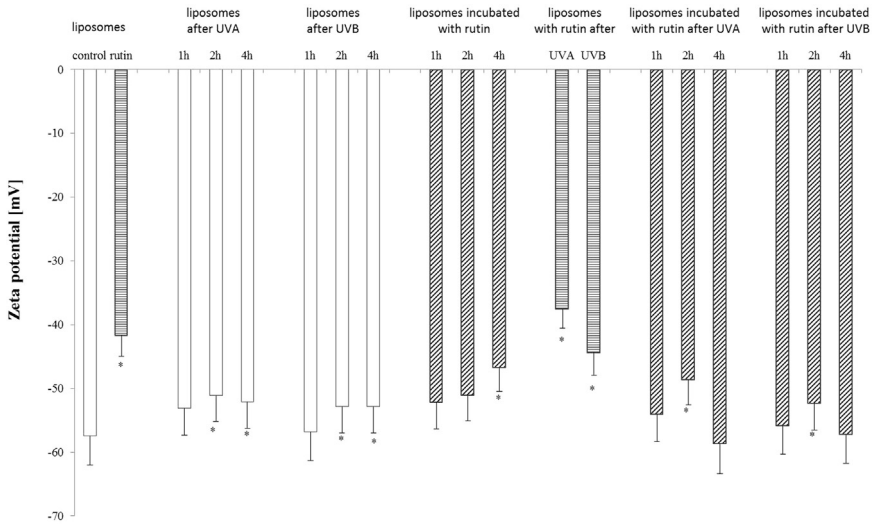

Fig. 7. Zeta potential of asolectin liposomes (clear, incubated with rutin [25 μM], synthesized mixed with rutin (1:1), and treated with UVA [20 J/cm2] and UVB [200 mJ/cm2] radiation). Mean values ± SD of five independent experiments are presented. * statistically significant differences vs. control liposomes, $p < 0.05$.

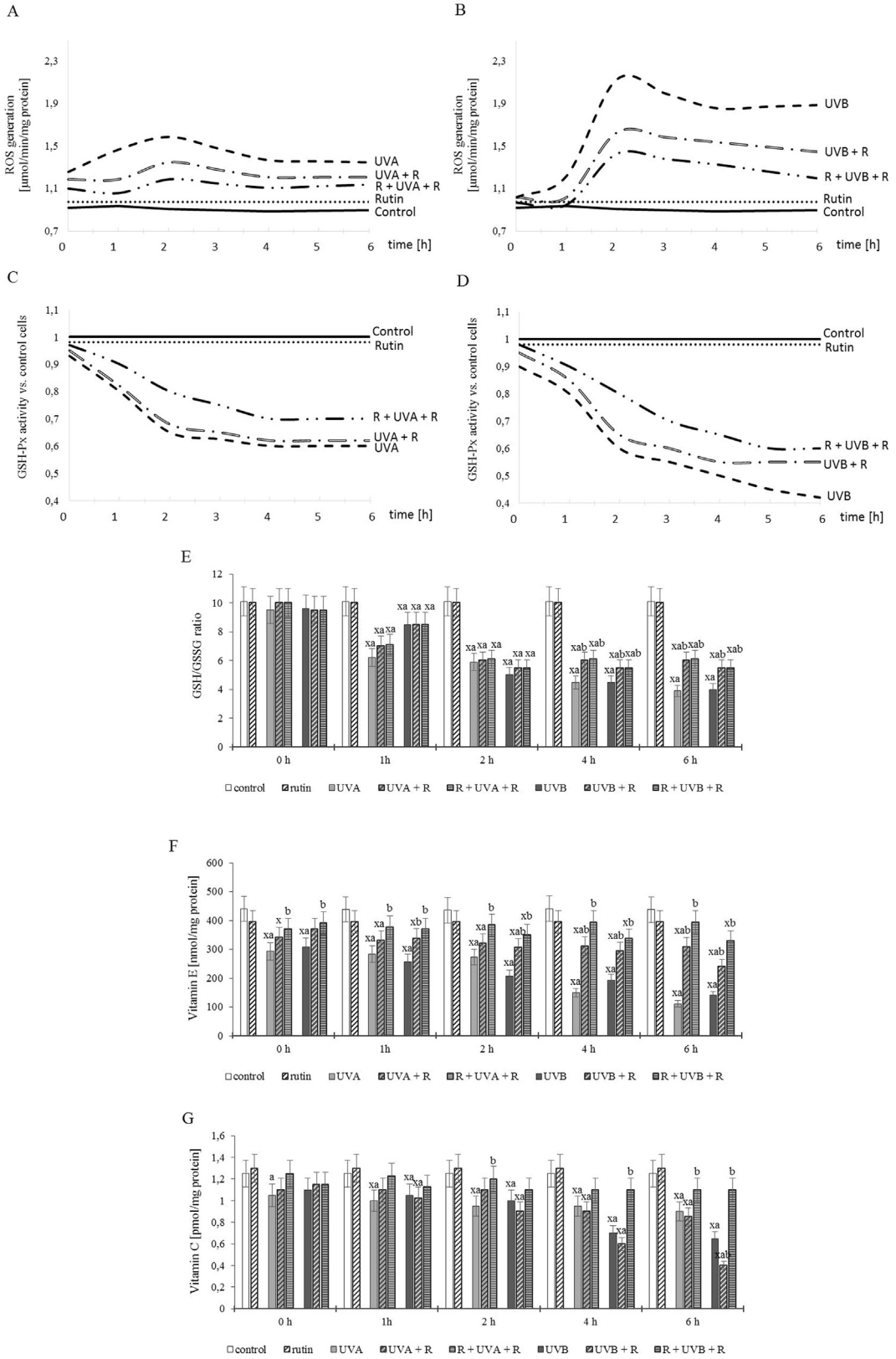

Fig. 8. Time-dependent changes in intracellular oxidative stress in fibroblasts following UVA [20 J/cm^2] and UVB [200 mJ/cm^2] radiation and rutin [25 μM] treatment and pre-treatment expressed as ROS generation (A-B), GSH-Px activity (C-D), GSH/GSSG ratio (E), and vitamins E and C levels (F-G). Mean values ± SD of three independent experiments are presented. ^{x} statistically significant differences vs. control group, $p < 0.05$; ^{a} statistically significant differences vs. rutin treated group, $p < 0.05$; ^{b} statistically significant differences vs. UV irradiated group, $p < 0.05$.

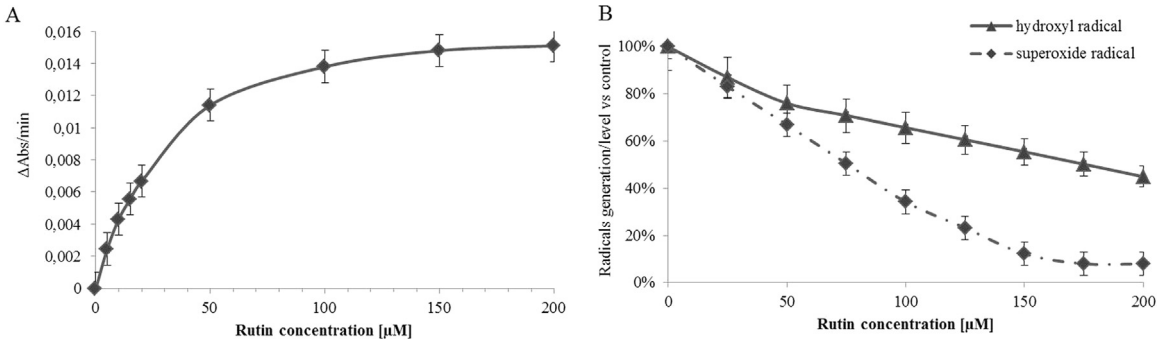

Fig. 9. Antioxidant activity of rutin [0–200 μM] measured in the reaction with ABST and expressed as a decrease in absorption at 734 nm per minute (A). Radical scavenging activity of rutin [0–200 μM] measured as the ratio of superoxide or hydroxyl radical generation/level in the control sample relative to that with rutin. Mean values ± SD of five independent experiments are presented.

4. Discussion

Human skin is the largest organ and acts as a physical barrier that protects the body from environmental factors. However, skin is also being increasingly recognized as a metabolically active organ. Skin is constantly exposed to environmental chemical and physical stressors, including UV radiation, and has therefore developed a wide variety of defense systems, the presence of which is believed to have evolved as an adaptive response to challenge by external factors [36]. However, constant exposure of human skin to UV radiation often requires additional external support in the form of compounds that prevent the metabolic consequences of UV radiation. A large group of natural compounds that have been proposed for this purpose are flavonoids. One of the biologically active flavonoids, rutin, is a quercetin glycoside. The protective effects of rutin on skin cells have been studied in the context of sunscreen enhancers, especially in association with UV filters [11]. The action of rutin on skin cell metabolism should be effective if it penetrates the fibroblast cellular membrane. However, the location of natural polyphenols, e.g., flavonoids, in the biomembrane remains controversial. Previous studies have indicated that flavonoids with numerous polar OH groups (such as rutin) are located deep inside of the membrane [37]; however, other studies have reported that flavonoids are located closer to the membrane surface, where they interact with the polar head groups of lipids via hydrogen bonding [38]. Therefore, the biological consequences of membrane penetration by rutin and its interactions with UV irradiation were the main objectives of this study.

This study shows that under physiological conditions, rutin only slightly penetrates the fibroblast membrane, while UV radiation significantly enhances rutin transport through the fibroblast phospholipid membrane. For topical application, skin permeability appears to be the major barrier; however, cells possess numerous transmembrane proteins that are responsible for xenobiotic transport [39]. The most accurately described transporter, bilitranslocase, operates as a flavonoid-specific membrane transporter; it is a membrane organic anion carrier that has been indicated in the liver, epithelia of the gastrointestinal tract and kidney [40] and whose existence was demonstrated in skin fibroblasts for the first time in this study. Our results indicated a significant upregulation by UV, particularly UVA irradiation, on bilitranslocase activity. The consequences of its upregulation, including the translocation of rutin from the medium into the fibroblast membrane and cytosol, are quickly observed. The partial lipophilic character of rutin results in over a 24-fold increase after UVA and 8-fold increase after UVB in the fibroblast membrane, while the cytosolic rutin level is only increased by approximately 15-fold after UVA and 5-fold after UVB irradiation compared to native translocation. Such conditions promote the fast protective action of rutin in both the cytosol and biomembrane following UVA irradiation. However, after 4 h, the level of rutin in the biomembrane is rapidly decreased, while the cytosolic level remains constant. Bilitranslocase translocates flavonoids according to their level [41]. Therefore, when the amount of rutin in the membrane is low, a transporter may carry it from the medium into the membrane, but when the level of rutin in the membrane reaches a sufficient level, bilitranslocase begins to remove the substrate. These observations are very useful because UVA radiation penetrates deeply through the epidermis into the dermis, while UVB operates mainly in the epidermal basal cell layer of the skin; however approximately 15% of UVB radiation also reaches dermal cells and induces direct and indirect adverse biological events [42]. Based on these results, it would be interesting to examine the actions of bilitranslocase following UV radiation in vivo.

Irrespective of this study, previous reports have indicated prolonged (after 24 h) protective effects of rutin on fibroblast metabolism during UV irradiation [19]. The results from the present study show that rutin may rapidly scavenge ROS and that such abilities are attributed to its chemical structure, which consists of three rings: two aromatic rings (A and B) and a hexagonal ring C connected by three carbon atoms [16]. During radical reduction, rutin is able to transfer one or two hydrogen atoms (mostly from the *ortho*-dihydroxyl group in the B-ring), resulting in the formation of quinonoid structures [43]. Rutin's antioxidant properties are determined by the presence of hydroxyl groups in the A ring, particularly at the C-5 and C-7 carbon, as well as the double bond at C-3 of the C ring [17]. Independent of its direct ROS scavenging

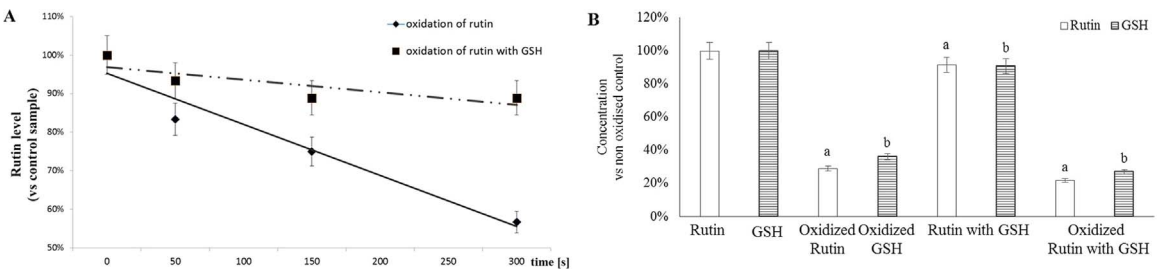

Fig. 10. Changes in rutin [50 μM] concentration following incubation with glutathione [40 μM] and oxidation by 1.6 nM HRP and 33 μM H_2O_2. [A – time-dependent changes measured 50, 150 and 300 s following oxidation, B – changes following 5 min of oxidation]. Mean values ± SD of five independent experiments are presented. [a] statistically significant differences vs. rutin, $p < 0.05$; [b] statistically significant differences vs. GSH, $p < 0.05$.

activity, rutin may also decrease their levels by inhibiting the main cellular enzymes responsible for the superoxide anion - initial ROS generation, such as xanthine and NADPH oxidases, as was observed after fibroblast UV irradiation [8]. Moreover, rutin may prevent ROS formation by chelating transition metal ions, mainly ferric and cupric, by participating in hydroxyl radical generation in the Fenton reaction [44].

The analysis of time-dependent changes in ROS generation in relation to 4-HNE level shows that rutin has also a direct effect on amount of 4-HNE, which generation is associated with PUFAs peroxidation. Rutin influence on UV-induced 4-HNE generation takes on added importance in the relation to multiple 4-HNE functions – as it was discussed earlier [45]. 4-HNE may interact with proteins by adducts formation, what changes the structure and function of diverse structural and regulatory proteins and enables 4-HNE to participate in multi-step regulation of cellular metabolic pathways, as well as antioxidant enzymes level and activity [45]. Increase in 4-HNE level inhibits protein synthesis, disrupts mitochondrial respiration [46] as well as leads to genotoxic activity and systemic oxidative stress characteristic for development of skin diseases like psoriasis or skin cancer transformation [47,48]. Therefore, showed in this study rutin antioxidant abilities resulted in restoring of UV-induced 4-HNE generation also indicate rutin capacity to skin cells protection against lipid peroxidation that is enhanced under stress conditions as well as photoaging [49]. It may suggest the possibility to use rutin in pharmacotherapy of skin disorders associated with enhanced lipid peroxidation.

The lipidomic approach revealed that UV radiation-enhanced ROS levels lead to structural changes in the fibroblast membrane, as manifested by alterations in the phospholipid profile. An increase in the amount of phosphatidylethanolamine and phosphatidylcholine with a concomitant decrease in sphingomyelin was observed. The UV-induced increase in phosphatidylethanolamine and phosphatidylcholine may indicate the upregulation of synthesis and activity of enzymes that participate in lipid metabolism [50]. Moreover, our results are consistent with those obtained in UV-irradiated human keratinocytes, in which a decrease in sphingomyelin and an increase in phosphatidylcholine were shown [51]; this was determined to be associated with the UV activation of the keratinocyte sphingomyelinase pathway, which hydrolyzes sphingomyelin to ceramide and phosphocholine, an intermediate of phosphatidylcholine [52]. In our study, we observed a decrease in four sphingomyelin species that could result in the generation of structurally corresponding ceramides. Ceramides act as second messengers in the cellular signaling pathways of inflammation, oxidative stress and apoptosis, as well as in all of the metabolic and pathophysiological pathways modulated by these states and events in an individual [56–58]. Most of these processes were significantly enhanced in fibroblasts after UV irradiation [8] and were significantly reduced after rutin treatment [19]. However, as shown in this study, inhibition of lipid metabolism caused by rutin suggests that rutin, as a singlet oxygen quencher, suppresses UVA-induced ceramide generation, similar to that observed for vitamin E [56]. Flavonoids reduce sphingomyelinase activity via the elevation of the glutathione level [57], which may respond to rutin activity according to this paper. Moreover, sphingomyelinases lead to an increase in metalloproteinase activity [58] and, in this way, destroy skin conditions, while rutin-induced metalloproteinase inhibition has been shown previously, although it has not yet been related to lipid metabolism [59]. Our results indicate that UV irradiation also enhances the activity of phospholipase A2 and the release of arachidonic acid, an activator of sphingomyelinase [60]. Experiments on the pancreas demonstrate strong inhibition of PLA2 and decreases of the selectivity of this enzyme selectivity caused by ROS-induced changes in the B-ring of rutin [61,62]. Therefore, rutin-dependent phospholipase A2 inhibition may be responsible for the decrease in sphingomyelinase activity and phosphatidylcholine level, as well as the higher level of sphingomyelins.

Regardless of the alterations in phospholipid profiles, UV radiation can also cause changes in their localization in the fibroblast membrane, as well as modifications to the lipid bilayer that may result in observed changes in the zeta potential. Our results indicate that under the influence of UVB radiation, a larger increase in the negative zeta potential of fibroblasts is reached than under the influence of UVA radiation. This corresponds with the enhanced level of lipid peroxidation, which leads to the generation of polar compounds as well as increases in the membrane dielectric constant and capacitance. One important consequence of this phenomenon is the alteration of transport particles across the membrane [63], and therefore, the response to rutin membrane penetration after UVB irradiation is less when the 4-HNE level is the highest. The above processes perturb the lipid asymmetry of the membrane by exposing phosphatidylserine to the outer membrane surface, resulting in additional negatively charged groups. The same effect is observed in the case of rutin, which significantly reduces the membrane potential, especially in the case of UVA radiation. Moreover, it was previously suggested that the location and orientation of flavonoids affect the membrane structure and function, including its fluidity [64]. The presence of the deprotonated glucuronide moiety strongly enhances the electrostatic interaction with the polar surface of the bilayer and the number of hydrogen bonds increases, mostly due to water molecules. The negative charge has another crucial effect since it also makes the passage through the polar barrier of the membrane difficult, as is observed following rutin treatment after UVB irradiation. Therefore, we hypothesize that rutin penetration through the fibroblast membrane is dependent on two mechanisms: bilitranslocase activity, which is significantly activated following UVA irradiation, and membrane structure disturbances by lipid peroxidation products, which are strongly induced by UVB irradiation.

Changes in the fibroblast membrane phospholipid profiles are accompanied by structural changes, resulting in modifications of their functions with altered cellular metabolism and cross-talk between cells, as well as their contact with environmental and exogenous compounds. The UV-mediated increase of the activity of fibroblast phospholipase A2, which realizes fatty acids from phospholipids, changes the fatty acids profile of the phospholipid structure and should increase the free fatty acids level. However, the observed decrease in the level of free PUFAs indicates that their metabolism is associated with the increase in UV-dependent ROS generation; this effect is also indicated by the enhanced activity of many other lipid metabolic enzymes, such as COXs and LOXs [65,66], which enhance ROS-dependent as well as enzymatic lipid oxidation. Rutin significantly suppresses UVB-induced COX-2 and iNOS expression in human keratinocytes in vitro, as well as in mouse skin in vivo [67,68]; therefore, its phospholipid protection results not only from its radical scavenging activity but also from its inhibition of enzymatic peroxidation [37,69]. The time it took to reach the maximum increase in ROS generation, particularly after UVB irradiation, responds by enhancing lipid peroxidation and consequently generates products of PUFA fragmentation, such as the reactive aldehyde 4-HNE, which is metabolically active [45]; this corresponds with the time it took to reach the highest amount of LDH leakage from fibroblasts, which confirms damage to the fibroblast membrane structure and function. The prevention of UV, particularly UVB, membrane damage and restoration of reactive aldehyde, as well as LDH leakage at a significantly lower level, indicates the effectiveness of the antioxidant activity of rutin. Therefore, rutin may be beneficial for preventing UV, particularly UVB-induced ROS generation and lipid peroxidation, i.e., events involved in pathological states, such as photoaging and skin cancer. It is important that rutin is characterized by its stability toward oxidation due to the blockage of the 3-hydroxyl group in the C-ring by a sugar moiety [70].

The protective action of rutin on biomembranes is also associated with its antioxidant properties, as confirmed in this study by its ability

to trap the ABTS cation radical. Moreover, rutin protects endogenous antioxidants that participate in preservation against ROS-dependent peroxidation of the biomembrane phospholipid, which is mostly associated with GSH-Px. This enzyme catalyzes the continuous reduction of lipid peroxides at the expense of reduced glutathione by its conversion into an oxidative form [71] that is enhanced by UV radiation. Moreover, glutathione participates in the removal of reactive electrophilic compounds, such as 4-HNE, whose level is enhanced after UV irradiation, as well as other compounds in reactions catalyzed by GSTs (enzymes with diverse substrate species that have been identified in human skin cells) [72]. It was previously observed that GST activity is also enhanced by UV irradiation [73]. This report shows that the GSH level is reduced in the presence of rutin in vitro under normal and oxidative conditions. This result may to attributed to the fact that flavonoids with a pro-oxidative catechol B-ring structural element, such as rutin, promote the formation of GSH conjugates [74]. This explains the simultaneous reduction of rutin level which promotes a decrease in the GSH level and then in GSH-Px efficiency. In this work, we show that rutin acts as both a free radical scavenger and as a GSH and GSH-Px protector, which occurs simultaneously after irradiation. Previous in vivo studies have shown that rutin treatment significantly attenuates reductions in the level and activity of GSH and GSH-dependent enzymes (GSH-Px and GSSG-R) in various rat models of diseases [75]. Moreover, rutin, because of its partial lipophilic but similarly as other glycosides less than α-tocopherols character may efficiently trap chain-initiating peroxyl radicals from the aqueous environment and cooperate with α-tocopherol to directly scavenge these species, as has been previously suggested according to data from lymphoid cell lines [76]. Conversely, the effects of rutin on cellular metabolism are also associated with its prevention of the decrease of other cellular antioxidant enzymes activities, such as SOD, CAT and GSSG-R, in different pathologies [77–79].

Obtained results suggest rutin as an effective skin fibroblasts protector against UV-induced changes in structure and functions of phospholipid membrane, what may be the starting point for searching of natural compounds based therapy against skin carcinogenesis or photoaging. However, multidirectional activity of rutin on other skin cells and their interactions as well as on whole skin must be also determined in conditions of UV irradiation.

5. Conclusion

In conclusion, UVA and UVB radiation affect rutin interactions with the fibroblast biomembrane in a different manner. Rutin membrane penetration is promoted by UVA-induced bilitranslocase as well as PLA2 activity, while UVB radiation enhances ROS generation and results in stronger phospholipid peroxidation and higher destabilization and membrane permeability, which facilitates its interactions with phospholipids.

Conflicts of interest

The authors have no conflicts of interest to declare.

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