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Research paper

Improvements of cellular stress response on resveratrol in liposomes

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ABSTRACT

Resveratrol (RSV) has proven potential in prophylaxis and treatment of various disorders mediated by free radicals and oxidative stress. RSV solubility, stability, and cytotoxicity must be regulated for satisfactory bioavailability. Here, RSV was loaded into liposomes, characterized by PCS and TEM and evaluated on HEK293 cell line by metabolic activity assay, electron paramagnetic resonance, and fluorescence microscopy.

RSV at 10 μM induced changes in cell metabolic activity and significantly improved antioxidative capacity. At 100 μM it showed concentration-dependent cytotoxicity. Oligolamellar liposomes with mean diameter 84 nm, polydispersity index 0.2, and zeta potential −40 mV showed high entrapment of RSV and rapid cellular internalization. Cell stress caused by UV-B irradiation diminished cell metabolic activity by 50%. RSV loaded into them showed no cytotoxicity at 100 μM and stimulated cellular metabolic and antioxidant activity levels to eliminate the harmful effect of the stress. Localization of RSV within liposomal bilayer is crucial for stimulation of cell-defense system, prevention of RSV cytotoxicity, and its long-term stability. In summary, evidence of different metabolic activity using free RSV and LIP–RSV is presented indicating that liposome-mediated uptake of RSV is more effective for improvement of the cell-stress response.

1. Introduction

Antioxidants have received a great deal of attention in recent years for their prophylactic and therapeutic potential for diseases in which the cell defense against reactive oxygen species (ROS) is compromised [1]. Externally supplied antioxidants neutralize ROS before they react with biological targets to reduce oxidative stress [2]. The emerging view is that many antioxidants are likely to exert additional beneficial cellular effects, through modulation processes at different biochemical levels including protein synthesis and signaling pathways [3]. Natural and synthetic sources are therefore being screened for novel antioxidants [4].

The polyphenolic compound resveratrol (trans-3,5,4′-trihydroxystilbene; CAS 501-36-0; RSV) has been found in more than 70 plant species, including human foods such as grapes, peanuts, and various berries and herbs. It is thought to serve as a phytoalexin, protecting plants against environmental stress and pathogenic attack [5]. Its relatively simple molecular structure enables free radicals overproduced in disease conditions to be scavenged and the redox signaling pathways of the cells to be regulated. RSV was shown to affect mitochondrial function and metabolic homeostasis through induction of genes for oxidative phosphorylation and mitochondrial biogenesis [6]. The chemopreventive effect of RSV is thought to be due to inhibition of quinone reductase 2 activity, which in turn, up-regulates the expression of cellular antioxidant and detoxification enzymes (such as catalase, quinone reductase 1, glutathione-S-transferase) to improve cellular resistance to oxidative stress [7]. Additionally, its ability to interact with receptors and enzymes gives rise to other biological effects such as suppression of growth, induction of differentiation, cell-cycle regulation, down-regulation of pro-inflammatory mediators, regulation of gene expression by affecting transcription factor activity, and up-regulation of death-inducing factors. Recently, studies on a variety of species showed that RSV increased activity of SIRT (a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylase) which resulted in improved cellular stress resistance and longevity [8]. Moreover, RSV is structurally similar to diethylstilbestrol which broadens its activity on estrogenic area. Thus, through its phytoestrogenic properties it can regulate the expression of hormone-dependent genes, such as the oncosuppressor BRCA1, in breast cells [9,10]. All of these actions are dependent on cell conditions and RSV concentration [5]. These potential therapeutic and prophylactic applications are, however, restricted by the low bioavailability caused by its physical properties. Polyphenols typically are largely metabolized and consequently have oral bioavailability of <10% [11]. Additionally,

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RSV has low water solubility and stability making its clinical success a formidable technological and medical challenge [12].

Liposomal formulations have been proposed as a means of improving the therapeutic efficacy of poorly bioavailable drugs [13], and examples of formulation containing antioxidants, such as Cu/Zn superoxide dismutase, vitamins C and E, N-acetyl cysteine, and glutathione, have shown promise for treatment of diseases involving oxidative stress [14]. Although the effects of RSV on the molecular biology of cells have been well documented, only our previous report [15] has described its formulation in liposomes. This showed that stimulation of cell activity was inversely proportional to the RSV dose. Fluorescence images of the treated cells demonstrated effective antioxidant activity of RSV at 10 μM, but toxicity seen as changes in cell shape, detachment, and apoptotic features at higher concentrations was observed [15].

Our hypothesis is that liposomes increase RSV biological activity and improve its protective effect on stressed cells. This study compares the effects of RSV-loaded liposomes (LIP–RSVs) to free RSV on cell metabolic activity and cell-redox system as well. Effects of RSV on metabolic activity were quantified by MTS assay, while electron paramagnetic resonance (EPR) method was used to define activity of cell-redox system. Internalization of liposomes within cells was visualized by fluorescent microscopy. Evidence of different mitochondrial activity using free RSV and LIP–RSV is presented indicating that liposome-mediated uptake of RSV is more effective for improvement the cell-stress response.

2. Materials and methods

2.1. Materials

Resveratrol (RSV; >99% pure), cholesterol, and dicetyl phosphate (DCP) were obtained from Sigma (Germany); ATX Tris buffer from Fluka (Germany); and enriched soy phosphatidylcholine (Phospholipon 90G, P90G) from Natterman Phospholipids (Germany).

2.2. Methods

2.2.1. Solubility testing

Twelve different amounts of RSV were dispersed in water (obtained concentrations varied from 5 to 70 mg/l) and stirred for 24 h. The concentration of RSV in the supernatant was determined by UV–VIS spectrophotometry at 306 nm (Hewlett Packard, 8453, Germany). The maximal concentration gave the solubility of RSV in water.

2.2.2. RSV sample preparation

Stock solution of 100 mM of RSV in ethanol was stored at −20 °C. Final working concentrations were prepared by diluting with culture medium. The highest concentration of ethanol used on cell culture was 1.1% v/v, which preliminary experiments had verified to have no effect on viability.

2.2.3. Liposome preparation

Empty liposomes (e-LIP) were prepared by suspending P90G (129 μmoles), DCP (36 μmoles), and cholesterol (26 μmoles) in 5 ml of Tris–HCl buffer, adjusting the pH to 7.4, then sonicating with a High Intensity Ultrasonic Processor equipped with a tapered microtip (Cole-Parmer, USA), until an opalescent dispersion was obtained. The pulse function of sonicating was used to inhibit heat build-up in the sample. RSV was loaded into liposomes (LIP–RSV) by adding RSV (6.5 μmoles) to the suspension mixture. Liposomal dispersions were first extruded through 100 nm filter pore size in a LiposoFast™ extruder (Avestin, Canada) to produce small oligolamellar vesicles, and then dialyzed against water using Spectra/Por® membranes (Spectrum Laboratories, Inc. USA) to separate the free RSV.

Fluorescently-labeled liposomes were prepared by adding coumarin-6 dye (3 μmoles) (Sigma, Germany) to the lipophilic components before sonication.

2.2.4. Liposome characterization

Size and polydispersity index (PI) of liposomes were determined by dynamic light scattering (DLS), while zeta potential (ZP) was measured by laser Doppler electrophoresis (Zetasizer nano-ZS; Malvern Instrument, UK), taking the mean of five measurements [15]. For determination of liposomes stability, the size, PI, and ZP of prepared liposomes were accompanied during their storage for at least two months. Vesicle formation and the morphology of liposomes were observed by transmission electron microscopy (TEM) (Philips CM 100 microscope; Amsterdam, the Netherlands).

The amount of RSV loaded into liposomes (Encapsulation efficiency) was determined by high performance liquid chromatography. Possibility of *cis/trans* conformation was considered. Separation was achieved on an XBridge C18 (4.6 × 150 mm, 5 μm) column by (Waters, USA) run on an Agilent 1100 Series HPLC System (Germany). RSV was separated using a mobile phase of 75% v/v methanol, 22.5% v/v acetonitrile, 2.4% v/v water, and 0.1% v/v acetic acid at 0.8 ml/min. Absorbance at 306 nm was recorded by a diode array detector.

2.2.5. Cell culture and treatments

Human-derived renal epithelial cells (HEK293 cells, American type culture collection ATCC, Manassas, VA, USA) were selected as model cells, since they can be easily manipulated. They were grown as adherent cultures in growth medium consisting of Dulbecco's modified Eagle medium (DMEM; Sigma, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco® – Invitrogen, USA), 1.0% 200 mM L-glutamine, and 1.0% antibiotic/antimycotic (Sigma, Germany) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

HEK293 cells were seeded at a density of 2×10^5 cells/ml over an appropriate growing area. After one day (attachment phase), the cells were treated for 24 h with (a) RSV at 10 μM or 100 μM, (b) empty liposomes (e-LIP), or (c) resveratrol-loaded liposomes (LIP–RSV) with concentration of RSV at 100 μM. Control cells received culture medium alone.

Some samples were exposed to UV-B radiation (UV lamp, Spectronics, New York, USA) at 280–320 nm for 1 or 3 h. Non-irradiated cells were kept in the dark in the incubator.

2.2.6. Metabolic activity of cells

Cell metabolic activity was evaluated using the MTS assay (Cell titer 96® Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). It is based on the capacity to metabolize of a yellow tetrazolium salt (MTS) to the dark blue formazan product by mitochondrial dehydrogenase. Briefly, after 24 h treating of cells, MTS reagent was added for 3 h and the finally the absorbance at 492 nm was measured. Metabolic activity of treated cells was expressed as percentage of untreated cells [15].

2.2.7. Morphological examination of cells

Cell growth and morphology were observed using an inverted phase-contrast microscope (Olympus CKX41, Tokyo, Japan). To establish fixed-slide preparations, HEK293 cells (2×10^5 cells/ml) were plated on square glass cover slips and incubated in six-well plates overnight. To visualize liposomes, the cells were incubated with fluorescently labeled liposomes. Following incubation with different test dispersions, the cells were fixed with ice-cold 4% paraformaldehyde in PBS (pH 7.4) for 10 min and permeabilized

for 10 min in 0.1% Triton X-100 (Sigma, Chemical Co., Saint Luis, ZDA). To visualize cells, the actinic fibres were stained with green-fluorescent dye Phalloidin–Fluorescein isothiocyanate or red-fluorescent Phalloidin–Tetramethylrhodamine B isothiocyanate (both from Sigma) according to the manufacturer's instructions. The red dye was used when fluorescent liposomes were added to the cells. Cell nuclei were then stained with DNA interchelating dye Hoechst 33342 (Riedel de Haen, Germany) (5 μg/ml) for 30 min in the dark. After staining, the cover slips were mounted on a slide and transmission micrographs and fluorescence images were collected simultaneously with the same focus settings (60- and 20-fold objective magnification) on an Olympus IX 81 fluorescence microscope and merged with Cell^R Software (Olympus).

2.2.8. EPR experiments

EPR spectroscopy enables the detection of cell-redox activity by the reduction kinetics of the nitroxide moiety of spin-label MeFASL (10, 3) (5-doxyl-methyl palmitate) that is incorporated in cell membrane [16] (Fig. 2). Cell-redox systems reduce nitroxide to non-paramagnetic hydroxylamine, which is not detectable by EPR. Thus, in metabolically active cells the EPR signal gradually decreases as reported by Swartz et al. [17].

Cells were treated as described in 2.2.5. After 24 h of incubation with 10 or 100 μM RSV, followed by UV-B irradiation for 1 h the cells were detached by thorough pipetting to yield a cell suspension. The suspension density was adjusted to 1×10^6 cells/ml and 3 ml was spin labeled with 75 μL 10^{-4} M of lipophilic spin probe MeFASL (10, 3) as follows: a thin film of MeFASL (10, 3) was spread on the wall of a glass tube by rotary evaporation of ethanolic solution. The cell suspension was added and the tube was gently shaken by hand for 10 min, centrifuged at 120*g* for 2 min, and the supernatant was carefully removed. For EPR measurements, the remaining cell pellet was drawn into a standard quartz capillary (1 mm diameter) and spectra were recorded using an X-band EPR spectrometer (Bruker ESP 300). Settings were microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 0.2 mT, centre field 340 mT, and microwave frequency 9.59 GHz.

The influence of RSV on the redox activity of the cells after 24-h treatment by RSV and then by UV-B irradiation was determined via reduction kinetics of the nitroxide group of the spin label. EPR spectra intensity (I) was measured with time after incubation of cells with the spin label. Signal intensity of the spin label is proportional to the number of the nitroxide groups. It can be calculated as the double integral of the EPR spectra and is approximated by Eq. 2:

$$I = h_0 \Delta H_0^2 \quad (1)$$

where ΔH_0 and h_0 are the width and amplitude, respectively, of the middle line of the EPR spectrum (Fig. 2C).

2.2.9. Statistical analysis

The values reported are means and standard deviations of experimental done in triplicate at least three times. Data were analyzed using one-way analysis of variance (ANOVA) and $p < 0.05$ was considered significant.

3. Results

The development of delivery systems that improve the biological profile of active ingredient is of utmost importance. This study reports how incorporation into liposomes improved the efficacy of RSV for prophylaxis or therapeutics. MTS colorimetric assay measures mitochondrial dehydrogenase activity in metabolically active cells, while the EPR method assesses the reduction of nitroxide-derived lipophilic radicals which have been implicated in the mitochondrial electron transport chain [18].

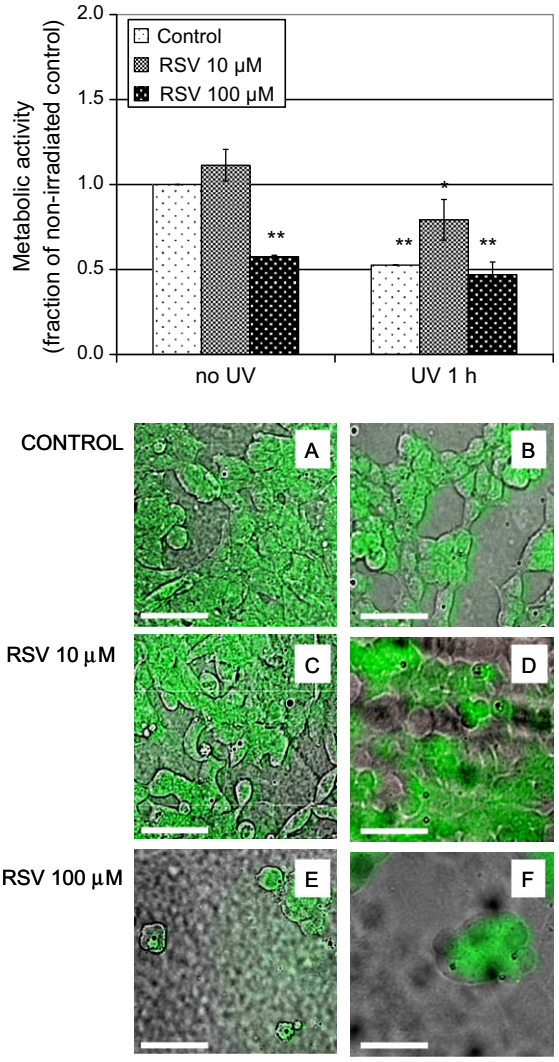


Fig. 1. Metabolic activity and morphology of HEK293 cells after treatment with resveratrol (RSV) and exposure to the stress condition. (Above) Bar charts show effects on metabolic activity after 24-h incubation of cells with medium (control), RSV at 10 or 100 μM, and further non-irradiated (no UV) or irradiated with UV-B irradiation for 1 h (UV 1 h). Calculations were made from the absorbance measured after addition of MTS reagent for 3 h relative to non-radiated control (mean ± sd; $N = 3$; $^{*}p < 0.05$; and $^{**}p < 0.001$). (Below) Fluorescent-transmission micrographs of cells (untreated (control) or treated with RSV at 10 or 100 μM) show green-stained actin fibres embedded by single cytoplasmic membrane. First column are non-radiated cells, while second column are irradiated cells. Bar is 50 μm.

3.1. Influence of RSV on cell metabolism

Mitochondrial dehydrogenase activity is a measure of cell viability, where a decrease indicates an arrest of cell proliferation [19]. Thus, the MTS assay evaluates the dose-dependent effect of RSV on cell activity under UV-stress conditions (Fig. 1).

RSV at 10 μM had no effect, while at 100 μM halved mitochondria activity. A similar decrease was caused by stress, triggered with UV-B irradiation. RSV at 10 μM partially protected against UV damage, but at100 μM there was no protection. These results suggest that RSV at higher concentration has a cytotoxic mechanism different from the UV-stress mechanism.

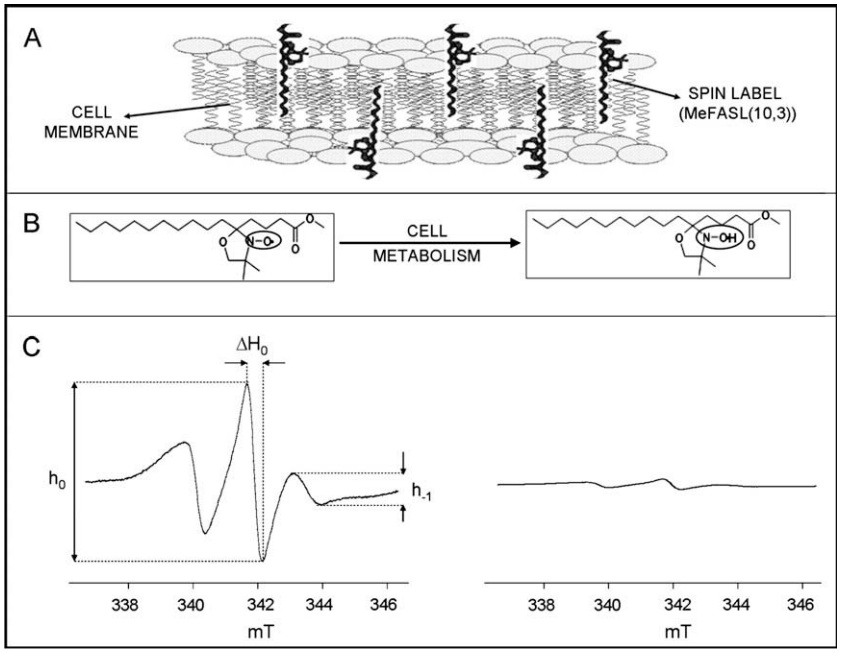


Fig. 2. Schematic representations of: (A) – distribution of spin-label MeFASL (10, 3) in the cell membrane; (B) – molecular structure of MeFASL and its non-paramagnetic hydroxylamine reduction product; (C) – diminution of EPR spectrum intensity of MeFASL during reduction by cytoplasmatic redox system of HEK293 cells.

Changes in metabolic activity are reflected also in cell growth and morphology, because all growth and divisions are enzyme controlled. Changes in these enzyme functions can be quickly reflected in cell confluence and shape. The empty areas in Fig. 1B show reduction of cell proliferation following UV-B radiation. RSV at 10 μM maintained the confluence under UV stress and preserved the shape typical of adherent cells (Fig. 1C and D). RSV at 100 μM reduced confluence and altered cell shape (Fig. 1E and F). The rounded and detached cells, which are indicative for necrosis or apoptosis, suggest cytotoxicity of RSV at 100 μM. This supports our previous findings, where fluorescence microscopy showed morphology changes and disintegrated actinic fibres after 24 h incubation with RSV at 100 μM, in both radiated and non-irradiated cells [15].

3.2. Influence of RSV on the cell-redox system

The protective effect of RSV on the cell defense mechanism was investigated. Influence of RSV and the effects of UV radiation on the cell-redox system were assessed by EPR using spin-label MeFASL (10, 3). The lipophilic nature of MeFASL (10, 3) localizes it in the cell membrane (Fig. 2A) [16], where it is bioreduced (Fig. 2B) and gives characteristic EPR spectra (Fig. 2C).

The ability of HEK293 cells to reduce MeFASL (10, 3) to the non-detectable hydroxylamine analogue (10, 3) after RSV treatment is shown in Fig. 3. EPR signal intensity was normalized relative to the intensity measured 4 min after cells exposure to MeFASL (10, 3). First order reduction kinetic characteristics of MeFASL (10, 3) are listed in Fig. 3C.

The reduction of radicals by the redox system of HEK293 cells is shown as a fall in signal intensity. Contact of cells with 10 μM RSV for 24 h stimulates the redox system which reduces free radicals. The gain for 10 μM RSV ($k = 6.26\ s^{-1}$; control = $4.94\ s^{-1}$; $p = 0.095$) is small in non-irradiated cells, but 10 μM RSV shows significant protection to irradiation ($k = 5.25\ s^{-1}$; control = $2.64\ s^{-1}$; $p = 0.01$). Radical elimination is even slower in the presence of 100 μM RSV, showing inhibition of the cell-redox system. The rate constants are presented in the Fig. 3C.

Redox activity was even more inhibited by UV radiation. Rate constants were lowered to 53% and 59% of non-irradiated but untreated and 100 μM RSV-treated cells, respectively. Student's *T*-test shows both are significant at $p < 0.05$. With 10 μM RSV the small decrease to 84% was significant only at $p = 0.12$, suggesting stimulation of the redox system at low concentration. The sequence of the rate constants in irradiated and non-irradiated groups was consistent with fastest reduction in RSV at 10 μM and slowest at 100 μM.

3.3. Comparison of metabolic activity determined by MTS and EPR assays

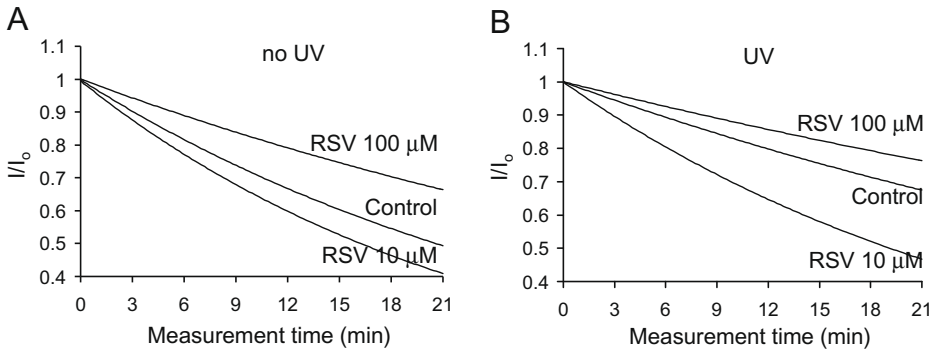
Results are expressed relative to those from non-treated or non-irradiated cells as appropriate (Table 1). Values from both, MTS and EPR results, are similar (lowest *p* value 0.24), and the trends are preserved throughout. Irradiated cells show a fall in activity to 56% in the MTS assay and to 53% in the nitroxide reduction. In non-irradiated samples both methods show an increase in cell metabolic activity in 10 μM and a decrease in 100 μM RSV.

Earlier studies on different cell types using lipophilic nitroxide spin labels revealed that they are not only localized in the plasma membrane, but are in dynamic equilibrium with other membranes [18]. Close agreement between the MTS and EPR assays points to an important involvement of the mitochondria in the changes provoked by RSV and UV radiation.

Our previous research has already shown the cytotoxic effect of 100 μM RSV as increased sub-G1 phase (apoptotic cells) [19]. Thus, RSV is a potent active substance with multiple antioxidative mechanisms that should be delivered to the cells in low dose. Ideally, RSV should be released slowly over a prolonged period from an appropriate carrier to avoid triggering cytotoxicity. This requires that liposomes need to contain an amount of RSV that would be toxic in its unloaded state to maintain therapeutic-free concentration.

3.4. Amelioration of biological effects of RSV by liposomes

Liposomes loaded with RSV (LIP–RSV) were prepared in order to improve RSV biological activity and stability and to diminish cyto-



C

	Rate constant (*k*) [(1/s) *10-2]	
	Non Irradiated	**UV-B Irradiated**
Control	4.94 ± 0.76	2.64 ± 0.57
RSV 10 μM	6.26 ± 0.57	5.25 ± 0.56
RSV 100 μM	2.93 ± 0.54	1.74 ± 0.33

Fig. 3. First order kinetics of RSV-mediated EPR signal reduction of spin-label MeFASL (10, 3) in HEK293 cells that were (A) – non-irradiated or (B) – UV irradiated. (C) – Table shows the rate constant (1/S) of nitroxide reduction curves. Cells were treated with medium only (control), RSV at 10 or 100 μM for 24 h, exposed to non- or UV-irradiation and spin labeled by adding 2.5 nmol of MeFASL (10, 3) per 10^6 cells. Subsequently, EPR spectra were recorded at the given time points. Reduction curves were normalized to the initial signal intensity.

Table 1
Cell metabolic and redox activities determined by MTS assay and EPR without (no UV) or with UV-irradiation for 1 h (UV). Cells were treated with medium only (control), RSV solution at 10 μM or 100 μM for 24 h. Values are reported relative to the result of non-treated and non-radiated control cells as mean ± SD; $N = 3$.

Condition	No UV		UV	
Metabolic-redox activity	MTS	EPR	MTS	EPR
Control	1.00 ± 0.00	1.00 ± 0.31	0.56 ± 0.00	0.53 ± 0.27
RSV 10 μM	1.11 ± 0.009	1.27 ± 0.23	0.84 ± 0.12	1.06 ± 0.23
RSV 100 μM	0.57 ± 0.01	0.59 ± 0.22	0.50 ± 0.07	0.35 ± 0.18

toxicity at higher total dose. Composition of liposomes enables to incorporate RSV, which expresses the lipophilic nature (log *p* of 3.1, water solubility <30 mg/l). Therefore, the entrapment efficiency was >70% and prepared liposomes were stable on storage. TEM photomicrographs and dynamic light scattering showed small oligolamellar vesicles with mean diameter of 84 nm and the polydispersity index of 0.2. The zeta potential was approximately –40 mV.

Metabolic activity and morphology of HEK293 cells after treatment with free RSV alone were presented in Fig. 1. Fig. 4 shows the corresponding results for high concentration of RSV delivered by liposomes and illustrates their potential as carrier for RSV stimulating the intracellular enzymatic systems. A significant increase ($p < 0.05$) in metabolic activity was seen in cells treated with e-LIP and LIP–RSV at 100 μM, which is in complete contrast to the drastic lowering caused by free RSV at this concentration (Fig. 1), showing that incorporation within liposomal bilayers suppresses RSV cytotoxicity at 100 μM.

MTS results revealed that "empty" liposomes, e-LIP, had no protective effect against UV-irradiation-inhibited metabolism comparing to non-irradiated samples (Fig. 4). LIP–RSV 100 μM fully protected the cells, since their metabolic activities were comparable to those of the non-irradiated controls. Furthermore, unchanged metabolic activity after more than 3 h of UV-irradiation demonstrated the possibility of prolonged protection (data not shown).

Unloaded liposomes gave no protection against UV-irradiation (Fig. 4D), and the unaltered cell morphology confirmed their biocompatibility (Fig. 4A and C). Treatment with LIP–RSV at 10 μM was unsuccessful (data not shown), but at 100 μM the growth area and cell morphology were unchanged (Fig. 4E and F), probably as a result of the slow, sustained release of RSV. Therefore, liposomes act as an appropriate, inert carrier system.

The last but not the least question of our research was if RSV diffuses through the cell membrane alone or it is carried into the cells by liposome uptake. Matthaus et al. used Raman microspectroscopy to show that liposomes enter cells by endocytotic processes [20]. In our case, localization of the liposomes was confirmed by fluorescently labeling liposomes which appeared as green dots dispersed throughout the cytoplasm around blue nuclei and embedded with red-stained actinic fibres (Fig. 5; e-LIP and LIP–RSV 100 μM). The cell morphology was unchanged illustrating their biocompatibility. On the other hand, actin fibres were degraded and hardly seen after addition of RSV 100 μM which pointed on its harmful effect (Fig. 5; RSV 100 μM). The results are supported with figures of cell population obtained by using invert microscope (Fig. 5; first column). It is shown, that cell confluence was similar between control cells and those treated with e-LIP and LIP–RSV 100 μM, while the cell growing area became rarely covered when RSV at 100 μM was presented in cell culture for 24 h.

4. Discussion

The influence of RSV delivered by liposomes on cell metabolic and antioxidant activity and its stress protective effect has been compared with free RSV. Liposomes clearly enable the carrier-mediated uptake of RSV by the cells and thus influence its intracellular fate.

Free radicals are ubiquitous in cells being generated by normal physiological processes, and their production is enhanced by UV-irradiation [21]. Because free radicals can cause cellular damage, organisms have evolved several defense lines. The most important

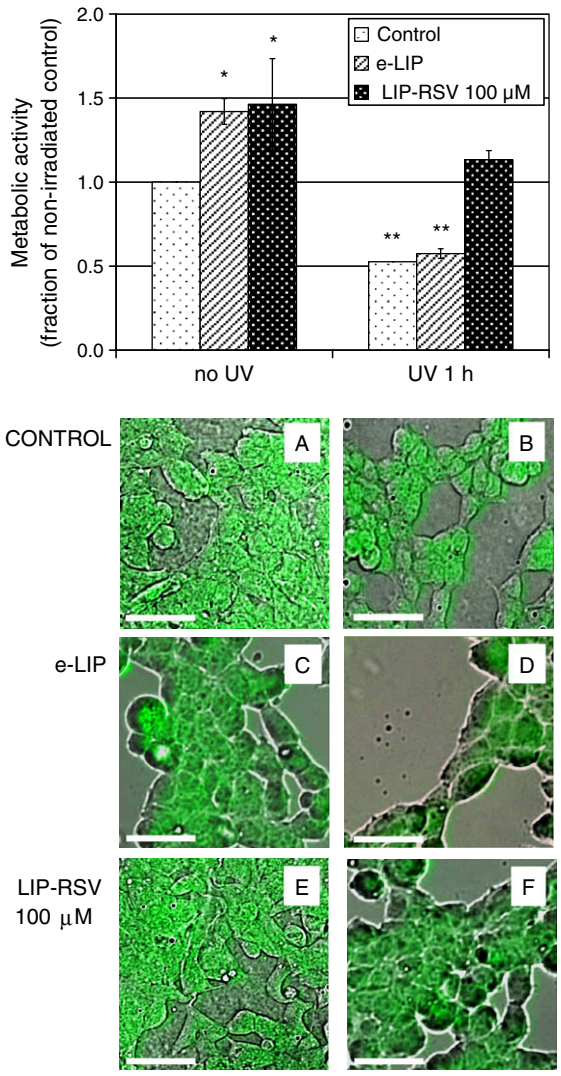


Fig. 4. Effects of liposomal preparations on metabolic activity and morphology of HEK293 cells treated with liposomal formulations and exposed to UV stress. (Above) Bar charts show effects on metabolic activity after 24-h incubation of cells with medium (control), empty liposomes (e-LIP) or liposomes loaded with RSV at 100 μM (LIP–RSV 100 μM) and further non-irradiated (no UV) or irradiated with UV-B irradiation for 1 h (UV 1 h). Calculations were made from the absorbance measured after addition of MTS reagent for 3 h relative to non-radiated control (mean ± sd; $N = 3$; $^{*}p < 0.05$; and $^{**}p < 0.001$). (below) Fluorescent-transmission micrographs of cells (untreated (control) or treated with e-LIP or LIP–RSV 100 μM) show green-stained actin fibres embedded by single cytoplasmic membrane. First column is non-radiated cells, while second column is irradiated cells. Bar is 50 μm.

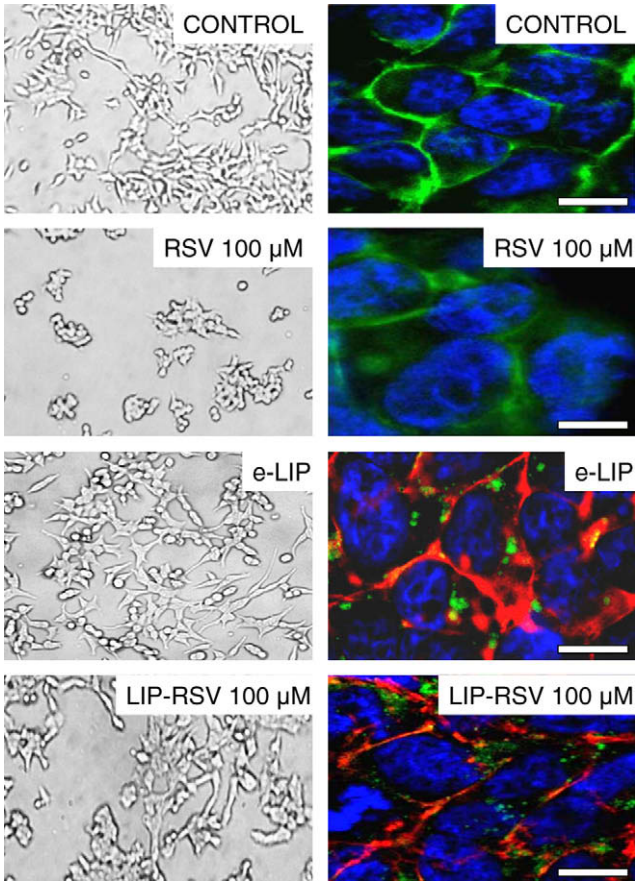


Fig. 5. Internalization of liposomes in HEK293 and their morphology after treatment for 24 h with medium (control), free resveratrol (RSV 100 μM), empty liposomes (e-LIP), or with liposomes loaded with resveratrol (LIP–RSV 100 μM). First column presents treated cells, pictured with an inverted phase-contrast microscope (Olympus CKX41, Tokyo, Japan), while pictures in second column were taken using the 60-fold objective on an Olympus IX 81 fluorescence microscope. On the pictures with "blue–green" fluorescence cells "nuclei-actin" are indicated, while with the "blue–red–green" fluorescence cells "nuclei–actin–liposomes" are stained. Bar is 10 μm.

are enzymatic (e.g. superoxide dismutase, catalase) and non-enzymatic (e.g. glutathione, vitamins E and C, ubiquinone) antioxidant systems [22]. An external supply of RSV is beneficial in overproduction scavenging directly and indirectly, influencing the cell-redox-signaling pathways via regulation of enzymes activity. All these processes are modulated via mitochondria [6].

Mitochondrial activity after RSV treatment and radiation-induced stress was assessed by EPR, for activity of the cell-redox system, and MTS assay, for mitochondrial dehydrogenase activity. These methods gave very similar results showing a dose-dependent effect of RSV on mitochondrial activity. Larger amounts of RSV triggered a cytotoxic effect, seen as lowered activity of dehydrogenase and the cell-redox system (Figs. 1 and 3).

RSV is known to upregulate endogenous antioxidant enzymes and other enzymes involved in protein replication and cell division [10]. RSV at 25 and 50 μM inhibited cell proliferation by cell cycle arrest in the G1/S phase [23]. The effect of RSV on growth and cell activity varied with cell type and depended on RSV concentration used and external conditions [10,24–28]. Interestingly, Stojanović et al. [29] observed that RSV is a better radical scavenger than vitamin E.

The effectiveness of antioxidants incorporated into liposomes has already been investigated by other researchers and shown to be appropriate delivery system for RSV for several reasons [14,30]. It is readily incorporated into their lipophilic bilayers because of its physical–chemical characteristics and transfer from them into the lipid domains of biological membranes. The amphiphilic properties of cell phospholipids enable further delivery to multiple intracellular sites as is shown in Fig. 5 and reported by Fang et al. [31]. Similar passive distribution of spin labels between the membranes and the uptake of the carrier into leukocytes was reported by Kristl et al. [32].

Localization of RSV into liposomal bilayers prevents transformation of the active *trans* conformation into inactive *cis* form (shown by HPLC analysis, 2.2.4). The –OH groups responsible for antioxidant activity are disposed at the liposomal surface for scavenging of radicals. Description of molecular orientation on the hydrophilic–lipophilic interface with respect to the chemical structure of compound can be found in the literature [33].

Finally, the liposomal bilayers store RSV and thus preventing overloading of the cell membranes. Its lipophilicity ensures that re-

lease of RSV into the cytosol is slow, which consecutively reduces its cytotoxicity. Liposomal bilayers offer the environment for RSV storage and thus cell membranes are prevented against high RSV loading, which caused deteriorative effects on the cells.

This study shows that RSV increases cell antioxidant capacity that can be consequences of direct scavenging action or the activation of the cellular pathways, which upregulate endogenous antioxidant enzymes. Nevertheless, liposomal formulation enables prolonged intracellular delivery of RSV with no cytotoxic response.

5. Conclusions

RSV is effective in protecting cells from free radical damage only when it is loaded into liposomes. Characteristics of RSV prefer its localization at the liposome surface, where it remains in the biologically effective *trans* conformation. Suchlike delivery system prevents the cells against cytotoxicity of RSV for long-term at 100 μM, controls its release and stimulates cell metabolic and antioxidant activities, which obviates the harmful effect of stress.

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