Resveratrol Modulates Oxidative Status in Rose Bengal Photosensitized Retinal Pigment Epithelial Cells

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Abstract

Eye exposure to high light intensities can produce a photochemical damage to retinal pigment epithelium (RPE), leading to severe pathologies. RPE D407 cells treated with Resveratrol (RSV, 100 µM, 24h) +/- photosensitizer Rose Bengal (RB, 500 nM, 1h) were exposed to green LED light. Cell viability and level of intracellular reactive oxygen species (ROS) produced after exposure to green light +/- RB were evaluated by MTT, respectively ROS assays. Spectrophotometric methods were used to determine GSH level and enzyme activities (CAT, SOD, GPx). Green LED light alone reduced cell viability with 30%, but a 40% reduction was observed in the presence of RB. RSV proved to have a strong positive impact, with a 20% increase in the viability of D407 cells exposed to green LED light. Viability of D407 cells exposed to green LED light +/- RB in the presence of RSV remained unchanged comparing to corresponding control. RSV blocked the intracellular ROS production, stimulated or restored the antioxidant enzymes activities and increased the level of reduced GSH in cells exposed to green LED light +/- RB. Our data came to support the potential use of RSV as protecting agent of retina’s antioxidant defence system in light-induced stress.

Keywords: antioxidant, photosensitization, resveratrol, RPE cells

Introduction

Lately, the human retina is more and more exposed to various types of light that shine everywhere in the developed countries. Light emitting diode (LED) is a source of light, especially in the liquid crystals displays of computers, TVs, smartphones and or tablets, which has been replaced the conventional light. When the eyes are exposed to light of high intensity in the visible range (390–600 nm), a common type of RPE damage called photochemical damage occurs. Green light (from 492 to 570 nm) has a long wavelength and is a part of the moderate-energy visible light spectrum. Previous studies performed on retinal photoreceptor cells suggested that white and green light are less aggressive than blue light (Grimm et al., 2001), (Kuse et al., 2014).

The photosensitizer is a critical component of photodynamic therapy, which can undergo two separate reactions. In the type I reaction, the exposure of photosensitizer to light determine the shift of an electron to a higher orbital, generating the triplet state. The type II reaction is the major cause of photochemical tissue damage, due to the production of singlet oxygen (and perhaps, of superoxide and hydroxyl radicals) following the interaction of photosensitizer with molecular oxygen and the return of the photosensitizer to the ground state (Pervaiz and Olivo, 2006), (Vermeersch et al., 1991). Therefore, maintaining
a constant presence of $O_2$ during irradiation of target cells furnishes a continuous production of singlet $O_2$. Singlet oxygen generated by this process is highly reactive, with a very short lifetime in biological systems (<0.04 microsecond) and an acting distance < 0.02 μm (Pervaiz and Olovo, 2006), (Vermeersch et al., 1991). Rose Bengal (RB, 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetrachlorodihydrofluorescein) is a hydrophilic photosensitizer, with very good singlet oxygen generation potential (Kochevar et al., 1996).

Since the retina is particularly vulnerable to oxidative damage and oxidative stress, the scientists attention has been drawn toward natural alternative therapies that could be used to reduce the damage of retinal pigment epithelium RPE cells induced by LED light, by modulating the oxidative status of the affected cells (Khandhadia and Lotery, 2010). Current research data demonstrated that resveratrol is able to suppress oxidative stress, by scavenging superoxide, hydroxyl, and other radicals (Leonard et al., 2003), (Pintea et al., 2011). Resveratrol (RSV) (3,5,4′-trihydroxystilbene) is a phytolalexin able to protect plants against injuries, stress, UV radiations or fungal infections. The protective effects of resveratrol have been intensively studied and its antioxidant effect is related to the up-regulation of the expression and of the activity of antioxidant and phase II enzymes (Pintea et al., 2011), (Mamalis et al., 2016). Also, resveratrol reduced the formation of light-induced carbonyl adducts in RPE cells exposed to UV-VIS broadband light (Lee et al., 2012). Beside its antioxidant potential, resveratrol was able to reduce the UBV-induced phototoxicity in RPE cells through the activation of SIRT-1, an NAD-dependent histone deacetylase which is down-regulated by UBV exposure (Chou et al., 2013). Recently, Koskela et al (2016) proved that resveratrol activates autophagy in RPE cells, a critical process for the maintenance of protein homeostasis in oxidative stress conditions (Koskela et al., 2016).

Therefore, the present study investigated the protective role of RSV in a model of photosensitization induced oxidative stress in retina cells.

**Materials and methods**

Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS) streptomycin, penicillin, amphotericin B were acquired from Invitrogen (Carlsbad, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Xenometrix (Allschwiil, Switzerland). Resveratrol (RSV) and SOD, GPx, CAT, GSH kit were taken up from Cayman Chemical (Ann Arbor, MI, USA). Hydrogen peroxide and all the others chemicals were acquired from Merck (St. Louis, MO).

**Cell culture and treatment.** D407 cells were maintained in DMEM supplemented with 10% FBS, 1 mM sodium piruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and were exposed to standard conditions: 37°C, 5% CO$_2$, and 95% relative humidity. D407 cells (6x10$^5$ cells/ 25 cm$^2$ flask or 1x10$^5$ cells/well, 96 well plate) were seeded and after reaching 80% confluence, the growth medium was replaced with fresh medium containing RSV (100 μM) and incubated for 24h. Afterward, the medium was replaced with complete media with or without RB (500 nM) for 1h. The exposure of treated D407 cells to green LED light exposure (2500 lux, λ=522 nm, 30 min) was done in Hank’s Balanced Salt Solution (HBSS) buffer.

**Viability assay.** The cellular model used in the current experiments was a D407 cell line (kindly provided by Prof. em. Dr.Dr.h.c. Horst A. Diehl, University of Bremen) which keeps all the human retinal pigmented epithelium characteristics. Cell viability was determined with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent. The method reflects the activity of mitochondrial succinate-dehydrogenase (Olchawa et al., 2010) . D407 cells (1x10$^5$ cells/well, 96 well plate) were treated with RSV+/- RB, and exposed to green LED light as described above. After 2h of incubation with MTT reagent, the excess reagent was removed and the formazan particles formed were solubilized with DMSO. The absorbance was read at 550 nm, respectively at 630 nm (for background) with a microplate reader HT BioTek Synergy (BioTek Instruments, USA). Cell viability was expressed as a percentage of control (untreated cells).

**Intracellular reactive species assay.** Cells were cultured in 96 well black plates and incubated with dichlorofluorescein diacetate (DCFDA), a non-fluorescent compound which can pass cell membrane, where is deacylated to 2′,7′-dichlorodihydrofluorescein
(DCHF). Intracellular reactive oxygen species (ROS) assay is based on the oxidation of 2',7'-dichlorodihydrofluorescein (DCHF) to the fluorescent compound 2',7'-dichlorofluorescein (DCF), which is measured by a microplate reader at excitation/emission wavelengths 488/525 nm, respectively.

Antioxidant enzymes and reduced glutathione assays. Cell extracts were prepared according with manufacturer’s instruction, and for each parameter the protein content was determined by the bicinchoninic acid assay (Sigma-Aldrich, DE). The activity of antioxidant enzymes was expressed as units per mg of total protein. The absorbance was monitored at 562 nm using a microplate reader HT BioTek Synergy (BioTek Instruments, USA).

Catalase assay. The assay is based on the peroxidation properties of catalase, catalysing the oxidation of methanol to formaldehyde, in the presence of $\text{H}_2\text{O}_2$. The resulted formaldehyde is measured colorimetrically, with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as chromogen, at 540 nm. Sample preparation and assay procedure were done according to kit instructions. One unit of CAT is defined as the amount of enzyme that will cause the formation of 1 nmol of formaldehyde per minute at 25ºC.

Superoxide dismutase assay. Superoxide dismutase (SOD) assay kit is based on the conversion of a tetrazolium salt to formazan by superoxide radical generated in xanthine/xanthinoxidase system. The protocol was followed according to the manufacturer’s instruction, measuring by this method, the activities of all three SOD types from the cell extracts. Absorbance was monitored at 460 nm. A standard curve made with bovine erythrocyte SOD was used for enzyme activity determination. One unit of enzyme is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Glutathione peroxidase assay. GPx Assay kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). The oxidized glutathione (GSSG) produced is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP$^+$ is accompanied by a decrease in absorbance at 340 nm. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP$^+$ per minute at 25 ºC.

Glutathione assay. Glutathione (GSH) assay was performed by an enzymatic recycling method with glutathione reductase. The sulfhydryl group of GSH reacts with DTNB (5, 5'-dithio-bis-2-nitrobenzoic acid) resulting the yellow 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. The absorbance was read after 30 minutes at 405 nm. A standard curve was done with GSSG standard.

Statistical analysis was done using Dunnett multiple comparison test of Graph Pad Prism version 6.00. Analyses were performed in triplicate, and experiments were repeated for three times. Values are mean ± SEM (*significant $P < 0.05$, **very significant $P < 0.01$, *** extremely significant $P < 0.001$) versus control (untreated cells).

Results and discussion

Protective effects of RSV on cell viability under photosensitization-induced stress

The positive effects of RSV (Fig.1A) on RPE D407 cells viability in photosensitization-induced stress model were measured based on the MTT assay. Rose Bengal (RB), a fluorescein derivative excited by green light, was used as photosensitizer in the current experiment. RB is currently used as stain in ophthalmic diagnostic procedures. As RB is unable to cross efficiently the cytoplasmic membranes, it is located mostly outside the cell (Olchawa et al., 2010).

The RPE D407 cells viability decreased with 30% in green LED light-induced stress conditions. Furthermore, exposure to RB photosensitizer (500 nM) dropped the cellular viability with 40% compared to control cells. According to data presented in Fig. 1 C, it can be seen that RSV (100µM) can improve with 20% the cell viability in green LED light-induced stress condition. However, when cells were exposed simultaneously to green LED light and RB photosensitizer, it was seen that RSV protective capability is reduced, the viability being comparable to that of the corresponding control (green LED light/RB). RSV proves its effectiveness only in the prevention of green LED light-induced stress. A study conducted on human retinal epithelial ARPE19 cell line showed that cells pre-treated with RSV (1-10 µM) had a better viability rate compared to the
ones simply exposed to UVA light, therefore RSV is effective in the prevention of UVA-induced ARPE19 cell damage (Chan et al., 2015). The characteristic morphology of D407 cells exposed to RSV treatment and to green LED light, in the presence or the absence of RB photosensitizer are presented in Fig.1B. Most of the cells maintain their physiological morphology, but the number of cells rounding-up and a detachment was observed in green LED light-induced stress in the presence of RB photosensitizer.

Interestingly, it was reported that photoreceptor-derived cells exposed to green LED light did not change their morphology, but they were affected by blue and white LED light (Kuse et al., 2014). The same study also reported that the changes in the morphology were in line with the decrease of cell viability, quantitative data showed a decrease of the cell viability by blue and white LED lights, but no influence of green LED light (Kuse et al., 2014).

Figure 1. Effect of resveratrol on the cell viability of D407 cells exposed to green LED light +/- RB photosensitizer. D407 cells were treated with RSV (100µM, 24h), +/- RB (500 nM, 1h). Chemical structure of resveratrol (RSV) (A). Phase contrast microscopy (20x magnification) on D407 cells exposed to green LED light-induced stress in the presence of RB photosensitizer after RSV (100µM, 24h) treatment (B). Cell viability was assessed using MTT test (C). Green LED light-induced stress in the presence of RB photosensitizer caused a significant decrease in cell viability, and this induced suppression was prevented by RSV treatment.

**RSVslessenedROSlevelinphotosensitization induced stress**

Therefore, our study investigated the intracellular level of ROS under photosensitization induced stress conditions and the protective role of RSV. Intracellular ROS level of RPE cells exposed for 30 min to green LED light was significantly increased compared to untreated cells. After adding RB photosensitizer, which has the capacity to attract and to absorb even more light into the cells, the ROS level increased with 30% compared to the ROS level of cells exposed only to green LED light. In both conditions, RSV treatment lessened the ROS intracellular level resulted after the stress induced by photosensitization. Comparing the intracellular ROS production induced by LED light in photoreceptor-derived cells, literature data attest that a more aggressive damage is produced by blue LED light than by white and green LED light (Kuse et al., 2014). Neutralization of ROS damage can be done either by scavenging of the radicals which are formed either by the inhibition of radical’s production. Another study showed that resveratrol was able to scavenge the free radicals but did not inhibit their production (Leonard et al., 2003).

![Figure 2: ROS production in D407 cells after green LED light-induced stress in the presence of RB as photosensitizer was suppressed by resveratrol (RSV). Representative histograms of DCF fluorescence intensity indicate the amount of ROS level in D407 cells treated with RSV (100µM, 24h) and +/- RB (500 nM, 1h) in green LED light-induced stress.](image)

**RSV increased the antioxidant enzymes activities in photosensitization induced stress**

As shown in Fig. 3, RSV increased the amount of the reduced glutathione and the activity of antioxidant enzymes: SOD and GPx treated with RSV (100µM, 24h) and +/- RB (500 nM, 1h) in
green LED light-induced stress compared with their corresponding control.

Superoxide dismutase (SOD) catalyses the dismutation of superoxide into \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \). RSV increased the activity of SOD with 1.2 units in the green LED light-irradiated cells, corresponding to its control. A more significant positive effect of RSV could be seen in green LED light-induced stress in the presence of RB photosensitizer; an increase with 4.1 units of the SOD activity was observed (Fig 3B). It was expected to have a dramatic elevation of SOD, due to its different subcellular locations in the cytosol and mitochondria, and a slightly elevation for CAT and due to its localization in peroxisomes in D407 cells treated with RSV (100\( \mu \text{M}, 24\text{h} \)) and +/- RB (500 nM, 1h) in green LED light-induced stress. CAT, respectively GPx are responsible to decompose \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \). Moreover, superoxide can be formed only during UV irradiation of RB and GSH, not after exposure to visible light (Menon et al., 1992).

RSV (100\( \mu \text{M}, 24\text{h} \)) had no significant effect on CAT activity in D407 cells exposed to green light, +/- RB (500 nM, 1h) compared with their corresponding control (Fig 3A). CAT proved to be involved in inhibition of RPE lysis upon UV-VIS irradiation in the presence of RB (Menon et al., 1992). An explanation for the different activities of CAT and GPx in RPE cells, in green LED light-induced stress +/- RB, could be related to the intracellular location and kinetic of these two enzymes. CAT is located in peroxisomes, therefore its access to cytosolic space is limited, meanwhile GPx is mainly present in cytosol and can be firstly saturated by \( \text{H}_2\text{O}_2 \) (Iloli-Assanga et al., 2015). RSV increased the GPX activity (with 8.2 units) in the green LED light-irradiated cells, while in the photosensitized cells (RB) exposed to green LED light the activity of GPX increased with 12.2 units, compared to their corresponding controls. The same tendency could be observed for the level of GSH in D407 cells pre-treated with RSV (100\( \mu \text{M}, 24\text{h} \)), in green LED light-induced stress compared to the level of GSH in photosensitized cells (RB) exposed to green LED light-induced stress.

In our previous study a significant increase of SOD activity was recorded for cells pre-treated with resveratrol, especially when cells were challenged with \( \text{H}_2\text{O}_2 \)-induced stress (Pintea et al., 2011). The positive role of RSV to up-regulate the protein expression levels and the activities of CAT and SOD1 on cancer PC-3, HepG2 and MCF-7 cell lines was reported, with no effect on the expression and activity of GPX1 in all tested cancer cells (Khan et al., 2013). The augmented activity of SOD in HaCaT cells exposed to UVA and treated with resveratrol could be responsible for the increased resistance to oxidative stress, proving that resveratrol could enhance the activities of antioxidant and phase II enzymes through the activation of NF-E2-related factor-2 (Nrf2) (Liu et al., 2011).

**Conclusions**

*In vitro* and *in vivo* (Jaadane et al., 2015; 2017) studies demonstrated that commercial LED light induce retinal injuries in which oxidative damages are involved. Enhancing the antioxidant status in the RPE cells might represents a strategy in limiting the damages produced by LED light sources (Jaadane et al., 2015; Jaadane et al., 2017). Our results proved that resveratrol treatment protects D407 cells against green LED light-induced stress, by preventing the decrease of cell viability, by reducing the intracellular ROS level and by modulating the antioxidant defense system. Further studies are needed to investigate the mechanism involved in the modulation of
intracellular antioxidant defense system by resveratrol.

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