Protective Effect of Green Light Against the Deleterious Effects of UV Irradiation on Cellular Systems

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ABSTRACT

A different type of experimental set-up is developed, in which cellular systems irradiated with ultraviolet (UV) light are protected through a simultaneous irradiation with high density green photons. The noxious UV-effects and their protection by green light (GL) are investigated on *Saccharomyces cerevisiae* (Sc) – cells, cultivated and measured under rigorous conditions. A series of physical determinations, atomic force microscopy, impedance spectroscopy and circular dichroism, revealed that the alterations induced by UV in the cellular culture were clearly reduced through the simultaneous GL irradiation. The biochemical activities of superoxide dismutase and catalase that “scavenge” free radicals in eukaryotic cells, severely damaged by UV, were almost completely restored by a previously protected GL- irradiation. The free radicals inhibition by high density photons, in the green band of visible spectrum, as a first revealed antioxidant effect of electromagnetic (EMF)-field, is analyzed in the context of a suggested tentative physical model.

Key words: free radicals, green light irradiation, protein polarization

INTRODUCTION

The significant damages induced by ultraviolet (UV) radiation at biological and environmental level turned lately into a debated social concern (1) and a highlight scientific medical problem (2). The noxious effects are discussed in biology by the oxidative stress and the free radicals generation. In chemistry a “free radical” is defined as an atom or molecule with an unpaired electron. One of the forms of reactive oxygen species (ROS) is the “activated” oxygen singlet state (O-O:), with the resulted oxidative damage, described in chemical lingo. At the lipids level chain breakage and cross-linkage, at the protein level fragmentation and aggregation, at the nucleic acids level deletions and mutations are the

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main described effects but a bio-physical mechanism underlying all these UV-damaging effects is still lacking. Eukaryotic cells have developed a series of defense mechanisms to cope with UV-aggression, mainly “scavenger enzymes” that neutralize free radicals. Regarding exogenous protection, our resources are scarce and less efficient, since we fight free radicals mainly with chemicals. Our group reported a series of different results in this field, revealing an antioxidant effect of electromagnetic field (EMF). We use the term different in the meaning of the physicist Philip Anderson (3) instead of the ubiquitous “new”, prevailing in modern scientific literature. A phenomenon/reaction/property is different when it places what is already known in an alternative perspective. The occurrence of EMF as a new player will change the relationship between the antioxidant effects already present. The present study focuses mainly on this difference. We developed a special experiment using green light (GL) irradiation sources (powerful monochromatic light-emitting diodes) that generate high-density green photons (HDGP). The protective experimental set-up represents UV-sample irradiation, perpendicular up-bottom, with a simultaneous GL-sample irradiation, perpendicular bottom-up. Our results revealed an antioxidant effect of GL on cells subjected to lethal doses of UV, proved on cell viability (4) and a clear GL-protective effect, proved on the structure of proteins subjected to denaturing doses of UV (5). We report here a chemical and physical investigation, as a new basis for the GL-protective effects, on a laboratory *Saccharomyces cerevisiae* (Sc) strain. A theoretical analysis of the biological and ecological implications raised by our experimental results is developed.

MATERIALS AND METHODS

UV and green light irradiation

2 mL cells (2×10^8 /mL) were irradiated on Petri dishes (ϕ 30 mm). The light beam was collimated to cover the entire surface of the target. UV irradiation was carried out using a Vilbert Lourmat UV lamp, model VL-204 G, 16 W, emitting at $\lambda = 254$ nm. A special geometry is arranged in order to deliver irradiation fluxes at $11.0 \text{ J m}^{-2} \text{ s}^{-1}$, in set-ups of 4 min exposure. The GL irradiation was carried out with 1 light-emitting-diode (16 V, 200 W, 1000 lumens, Ever Red Tronics, E20 Wg 120C) mounted on a copper ventilated radiator. A collimated monochromatic light $\lambda = 520$ nm with 15 mW/cm intensity, 95 mV incident on the target area was used. The protective experimental set-up represents UV-sample irradiation,

perpendicular up-bottom, with a simultaneous GL-sample irradiation, perpendicular bottom-up.

Strains and culture conditions

The *Saccharomyces cerevisiae* strain used in this study BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) was obtained from EUROSCARF (www.euroscarf.de). Strains were grown in standard YPD or SD (synthetic dextrose) medium supplemented with the necessary amino acids. Overnight pre-cultures were inoculated in fresh media at density 10^6 cells/mL, then grown with shaking (200 rpm, 28°C) to midlogarithmic phase (5×10^6 cells/mL) in SD medium. Cells were washed with 0.1 M MES/Tris (2-N-morpholino ethanesulfonic acid/2-amino-2-hydroxymethyl-propane-1,3-diol), pH 6.5 and resuspended in SD medium to 2×10^8 cells/mL. Cell growth was checked at time intervals by determining optical density of cellular suspension at 660 nm (Shimadzu UV-vis spectrophotometer, UV mini 1240).

Atomic force microscopy

20 μL of cellular suspension, 10^8 mL^{-1} in SDC (synthetic dextrose complete) medium, were placed on a quartz plate and slowly evaporated at room temperature. The tapping mode was selected as the most suitable for imaging of soft biological samples. The AFM- NT-MDT NTEGRA Prima instrument was used.

Impedance spectroscopy

A Zahner IM6 Khronach (Germany) electrochemical unit with $<0.0025\%$ frequency stability was used, with a miniaturized electrochemical cell (50 μL) with two platinum electrodes bands (1mm width, 0.1 mm thickness). The Cole-Cole plot reveals the dependence at low values for the in-phase and out-of-phase components of impedance. The main parameters are R_{Ω} -solution resistance, R_{ct} -charge transfer resistance and C_d -double layer capacity.

Circular dichroism

Measurements were performed on a Jasco J815 dichroism equipment, using a water-cooled thermostated Peltier device with 0.1°C accuracy, in the 163-900 nm range, on Hellma Suprasil quartz cuvette 0.1 cm path, with a 10^8 cell solution in deionized water. In order to obtain the dichroism signal from intracellular proteins of cells with a known hard membrane as

Sc-cells, the cellular culture was previously immersed in 10 mL bi-distilled water for 10 minutes.

Enzyme assay

For enzymatic assays, cells were washed with 0.1 M MES/Tris, pH 6.5, and concentrated to 1×10^9 cells/mL. Aliquots (100 μ L) were prepared in 1.5 mL tubes, and cell lysis was achieved with acid-washed glass beads (Sigma) by vortex mixing (3 min, 4°C). Cellular debris was removed by two centrifugation steps (10,000 rpm, 2 min, 4°C). The supernatant was collected after each spin, and the resultant crude lysate was assayed. Enzyme activity was normalized to total cell protein, determined by Bradford method (6). SOD (superoxide dismutase) - activity was determined as described in (7). CAT (catalase) – activity was determined using Sigma Catalase Assay Kit. The hydrogen peroxide substrate remaining after the action of catalase is checked spectrophotometrical at 520 nm. Glyceraldehyde-phosphate dehydrogenase (GPDH) was determined as described in (8).

RESULTS

Atomic Force Microscopy (AFM) is a powerful technique used to obtain images of cells at high resolution. The technique may uncover physical properties in the cell walls surroundings, as well as subtle biochemical details concerning cell walls polysaccharide surface and inner layers microtubules protein structure. We have applied AFM for revealing the Sc-cell wall transformation induced by UV-irradiation and by GL-protection. The measurements were performed on evaporated cell suspension at room temperature in air, according to the method used for electroporation (9).

Cell membranes act as physiological barriers to the surrounding medium. Extensive studies on the bilayer structures in the cellular proteins walls revealed complex mechanisms linked to the liquid contact with the bilayer, the location close to the hydrophobic bilayer core and the asymmetry in density distribution as result of molecular dynamics. Cellular water, as a polar molecule, may cross the cell membrane by osmosis. Due to this process, in our control samples, under the slow room temperature evaporation water is excluded by diffusion outside the cell. As a consequence deflections are observed at the cellular walls level (*fig. 1a*). Under UV-irradiation, the membranes inner layers proteins are unfolded, through a well-studied biochemical mechanism. The unfolded process grows progressively, devoid of ruptures. Therefore the surface

Figure 1- Effects of Sc-cells irradiations with UV and GL.
a) AFM- 3D image of a control sample. Under evaporation the water leaves the cellular structures, creating holes in the cells surfaces. b) AFM- 3D image of an UV irradiated sample. UV irradiation will preclude water evaporation (liquid holding recovering kinetics) due to the cells membranes protein modifications. The cellular surfaces appear swollen and rounded. c) AFM- 3D image of an UV+GL irradiated sample. The cellular structure under GL protection exhibits a water depleted surface close to the control

of the UV-irradiated sample appears smooth and rounded (*fig. 1b*). This interpretation of the proteins unfolding mechanism is consistent with the CD measurements, performed in similar experimental conditions. Under GL, the membrane inner layers proteins are only partially unfolded, leaving more cellular canaliculi open (*fig. 1c*).

Our results correlate with the now classic observations of Fröhlich, concerning coherent electrically polar longitudinal vibration in biological systems (10). These

vibrations may generate endogenous (EM)-fields, that recently have been detected in a broad frequency spectrum. Coupling the electromagnetic (EM)-fields with the UV and GL photons may generate energetic disturbances, resulting in local nanomechanical displacements. Pelling (11) detected these phenomena on Sc-cell membranes by AFM, a result that may be connected with our observed AFM- cell membranes modifications.

Electrical Impedance Spectroscopy (EIS)

Recent advances in impedance spectroscopy, based on cellular technologies, have provided physical type of information concerning cellular long-term behavior (12). We used electrical impedance spectroscopy (EIS) to reveal transport properties of ions across cellular membranes, at a fixed applied potential in the low frequency domain (100 KHz). The involved model considers cell membranes as RC-circuits and the internal medium as cellular resistance. Under UV-irradiation the complex impedance ($Z^*=Z'-iZ''$) of control sample decreases significantly, as the charge current increases. This is the effect of free radicals generation under UV irradiation. During GL-irradiation the complex impedance takes intermediary values, revealing a tendency to restore normal conditions (fig. 2), as a consequence of the antioxidant effect of GL.

Table 1- Impedance parameters for the UV and UV + GL samples

Impedance (Ω)	Sample	UV	UV+GL
R_Ω	928	894	905
$R_\Omega+R_{ct}$	987	952	963
$R_\Omega+R_{ct}-2\sigma^2C_d$	1023	975	1003
$R_\Omega+R_{ct}/2$	957	923	934

Since our results reveal differences in the low frequency domain, the electrical processes are due mainly to membrane transport property and less to the intracellular environment (table 1).

We investigated also the electrical impedance of the cellular lysates, irradiated with GL, in order to detect the protein structure modifications that may account for the recorded UV-protective effects. We represent the results by the conventional Bode plot: the absolute value of the impedance ($|Z|$) and the phase shift (θ) are plotted with log frequency. A sharp decrease of cellular lysate impedance is observed after GL- irradiation (fig. 3a) as well as a net modification of phase angle (fig. 3b) indicating a shift toward a pseudo capacity behavior, due to the orientation of polarized proteins on the electrode surface. All these results, well corroborated with the circular dichroism data, clearly suggest the protein polarization effect under GL - irradiation

Figure 2 - Impedance plots in the complex plane for Sc-cells suspension. The inset represents the complex impedances of control sample, UV and UV+GL irradiated cells samples. Impedance values for the UV+GL irradiated cells show intermediate levels, between the control sample and UV irradiated cells, revealing a protective effect of GL against UV-irradiation

Figure 3 - a) Bode diagram: impedance modulus vs frequency of Sc-cellular lysate under GL- irradiation. b) Bode diagram: phase vs frequency of Sc- cellular lysate under GL- irradiation

(generation of P^* - polarized protein as reported previously (13).

Circular dichroism

Circular dichroism determines the difference in absorbance between the L (left) and R (right) circularly polarized light components [$\Delta A = (A_L - A_R)$] reported in terms of ellipticity (θ) in degrees, ($\theta = \tan^{-1}(b/a)$), where b and a are the minor and major axes of the resulting ellipse, with $\theta = 32.98 \Delta A$. The main unit used in proteins studies is MRE (mean residue ellipticity), the molar ellipticity of the molecules divided by the number of monomer units, computed as $MRE = \theta/10 \text{ r}$, where θ is the recorded CD (mdeg) r - the number of amino-acids residues and l - the path length (cm) of the specter cell. From it the α -helix content may be computed by referring the mean residue ellipticity for a helix of r -amides to the mean residue ellipticity of an infinite helix, or alternatively by fitting the recorded CD - curves with a specific Provencer-Glockner soft. This is the standard technique to analyze protein CD-spectra.

Conformational changes under UV-irradiation and (UV+GL)- irradiation are reflected in the CD spectra of yeast cells. These spectra exhibit a negative band at 224 nm characteristic for the α -helix structure and a positive band at 197 nm due to π - π^* transition. The UV - irradiation induces an unfolding process altering the geometry of polypeptide backbones and accordingly decreases ellipticity. Clear differences were observed, particularly within the regions of 195 nm and 222 nm (fig. 4) recorded.

For evaluation of the yeast sample circular dichroism the CONTIN3 method was used. This algorithm uses ridge regression to fit a linear combination of spectra of

Figure 4 - Recording of circular dichroism in Sc-cells UV - irradiation induced a cellular protein unfolding with a decrease of π - π^* and n - π^* transitions. The MRE is drastically reduced. The protection effect of GL is clear

known composition to match a spectrum of unknown composition. The CD spectra of 16 proteins whose secondary structures are known from X-ray diffraction data.

The protein secondary structures derived from this fit are alpha-helix, beta-strands and turns. The typical standard deviation for this fit is 0.06.

In our case, the MRW for the yeasts is 107.13 Da, sample concentration $C = 0.0012 \text{ mg/ml}$ with 171 residues. With these data we present the CD fitting for the (UV+GL) irradiated cells (fig. 5a) and the CD fitting for the U-irradiated cells (fig. 5b). The fractional composition of cellular lysate secondary structure in terms of alpha-helix, beta-sheet, turns derived from the respecting fittings are listed in the table 2.

Figure 5 - a) The CONTIN3 - fitting for (UV+GL)- irradiated Sc - cells.
b) The CONTIN3 - fitting for the UV- irradiated Sc - cells.

In the control cells, the recorded α -helical content was 44.7%, β -sheet 19.6%, turns 21% and unordered structure 19%. UV irradiation induced an irreversible denaturation process with a higher loss of α -helix content, from 44.7 % to 18.9%, as compared with control values. In the UV+GL combined irradiation the cells presented a smaller reduction in the α -helical content from 44.7% to 27.9% with an unchanged β -sheet content.

The small decrease in the α -helical content, recorded in the (UV+GL) - irradiated samples, suggests a more compact and rigid conformation of the GL - protected yeast cells.

Cellular antioxidant defenses include several enzymes, capable of removing oxygen radicals and their products. Sc - cells possess two main intracellular such "scavenger" enzymes.

- *Superoxide dismutase (SOD)*, mitochondrially located, MnSod (encoded by *SOD2* gene) and cytoplasmically located Cu/ZnSod (encoded by *SOD1* gene).
- *Catalase (CAT)* catalyses the breakdown of H_2O_2 to O_2 and H_2O . Sc- cells have two catalase types: A, peroxisome located (encoded by *CTA1* gene) and cytosolic located, T protein (encoded by *CTT1* gene).

Table 2 - The fractional composition of cellular lysate secondary structure

Cells	α -helix	β -sheet	Turns	Unordered
Control	0.447	0.196	0.21	0.191
UV	0.189	0.124	0.180	0.284
GL+UV	0.279	0.194	0.191	0.341

SOD is involved in a dismutation reaction in which a single reactant is converted into two different products. The oxidized SOD_{ox} reacts with the O_2^- - superoxide ion to form O_2 and the reduced enzyme SOD_{red} . This in turn reacts with a second superoxide and two protons to generate hydrogen peroxide and regenerate SOD_{ox} . A coupling with CAT achieves a second dismutation, converting hydrogen peroxide in H_2O and O_2 . In this context, our GL - protection experimental set-ups reveal an interesting biochemical result. Under UV-irradiation both "scavenger" enzymes are drastically inhibited. The decrease of SOD activity induces a decrease of H_2O_2 production and, by consequence, a severe decrease of CAT activity through low substrate enzymatic inhibition (fig. 6). Catalase performs another type of "protecting" reaction, through a peroxidative mechanism, reshuffling the toxic compounds (phenols, formic acid, alcohols) derived from the debris generated by the powerful UV-oxidation: $H_2O_2 + RH_2 = 2H_2O + R$.

The UV inactivation on SOD and CAT enzymes may be interpreted from a biochemistry viewpoint, by the modification of protein structure resulting in the loss of enzymic activity.

Our experimental results derived from AFM, impedance and circular dichroism all indicate a protein unfolding, inducing a modification of the enzymatic activity. The GL - irradiation through its protective activity may restore the native architecture and most of the lost enzymatic functions.

This is a different, significant biological effect, particularly with respect to the large value of the difference between the UV- denatured and the GL-protected samples. As far as we know this is the first reported result in which the activity of an enzyme,

Figure 6 - Enzyme activity of superoxide dismutase (SOD), catalase (CAT) and glyceraldehyde-P-dehydrogenase (GAPDH) under UV-irradiation and GL-protection

several damaged by UV-denaturation may be completely restored by a physical technique. A series of medical developments, derived from the “free radicals scavenger” property of GL may be considered, particularly for viral and degenerative diseases. Noninvasive explorations, with optical fibers centered on the green band in visible domain, are easily conceivable in the modern medical technologies.

DISCUSSIONS AND CONCLUSIONS

This paper presents a series of different experimental results with practical as well as basic implications. The antioxidant proprieties of green photons may find large practical perspectives. The cellular protection of specific biological specimens may have important applications in regenerative medicine.

At the basic level the antioxidant effects of EMF raise a series of fundamental questions connected with biochemical energetics. The canonical knowledge of cellular energy generation, storage and subsequent intra/extra- cellular migration, is mainly based on the chemical potential concept. The introduction of the field notion, with all its powerful theoretical and experimental techniques, brought up by the up-to-date physics, may open new research avenues in this domain. Hence the different nature of our approach.

It is well known that the UV - light has an oxidant effect on the living cells, producing free radicals. Nevertheless this significant effect, with large biological as well as ecological implications, is presented only in qualitative

biochemical language. A rigorous biophysical/physical discussion is still lacking. In this context, our data open new perspectives in this direction.

The protective effect of intense GL against UV-induced oxidation observed in this study appears different since, under normal conditions, most biological structures (with the exception of green light receptors, not studied here) do not/weakly absorb green light. Therefore, the protective effect cannot be discussed in terms of electronic excitations. Moreover, the observed protective effect becomes effective after several minutes (20-30 min) of GL - irradiation, therefore the scales involved here are much larger than the corresponding electronic excitations (nanoseconds or shorter). To explain the protective effect of GL, against oxidation through UV-light, we suggest the hypothesis that electric polarization effects are responsible for the observed experimental results.

As known from literature, very large macromolecules may exhibit different metastable configurations with conformational transitions between them (15). These type of large molecules are generally considered as metastable states (16, 17).

It is likely that GL, with its lower photon energy may induce local polarization of the medium, with important consequences for the cohesion forces leading to tighter bound states with lower energy. These type of processes are well-known and studied (18).

The oxidant effect of UV-light is understood by the photopolarization of electrons, leading to free radicals, since the energy levels in the affected biological struc-

tures match the UV-photon energy. It is also known that the GL is very weakly absorbed, in normal conditions, by the biological structures, de-voided of green light receptors, which indicates an absence of electronic-transition processes. It is then likely that GL, (as well as longer wavelength light) with its lower photon energy may induce local polarization of the medium. This may have important consequences for the local cohesion forces, with changes in the electronic structure, leading to a tighter bound-state with lower energy. The relevant highest occupied molecular orbitals (HOMO) may get lower through such re-arrangement, so that the disruptive effects of UV-light are diminished. This may also explain the anti-oxidant effect of GL- as recorded in our experimental results. It is worth noting that such effects of the GL are effective especially over relevant lengths of the biological structures comparable with the GL-wavelength, so that, although similar protective effects may be expected from longer-wavelength photons, these are reduced appreciably. In our previous studies (19) we have confirmed experimentally this theoretical prediction- irradiations with blue and yellow recorded lower values.

When a biomolecule is irradiated with green light, whose electric field component oscillates with a frequency of 514×10^{14} Hz, the electronic clouds of the atoms are distorted and oscillate on that frequency with respect to the positively charged nuclei. As a consequence electric dipoles are induced on the atoms and the permanent dipole moments of the biomolecule (in our case the peptide groups of cellular proteins) are modified.

As a result the Coulomb- and dipole interactions inside the biomolecule are changed, which may drive an adjustment of the secondary and tertiary structure in order to minimize the electrostatic energy. A modification of the surface charge distribution may occur.

The polarization of the molecules with changes in the surface charge distribution may induce modifications of their interactions in solution, leading to the formation of macromolecular structures (clusters, macromolecular chains).

This aggregation process may lead to the stabilization of the tertiary and quaternary structures into a more rigid conformation, stable and less sensitive to disturbances.

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