Mitochondrial Alterations Induced by 532 nm Laser Irradiation

P. Kaššák[1], T. Przygodzki[2], D. Habodászová[1], M. Bryszewska[2] and L. Šikurová[1]

[1] *Division of Biomedical Physics, Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, Slovakia*
[2] *Department of General Biophysics, University of Lodz, Lodz, Poland*

Abstract. Mitochondrial alterations were monitored after low power green laser (532 nm, 30 mW) irradiation in the case of whole cells (B-14) and isolated mitochondria (from Wistar rat heart). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay products were significantly higher (by 8 %) in irradiated B-14 cells as compared to non-irradiated controls. Mitochondrial transmembrane potential of B-14 cells, measured by means of a fluorescent probe 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), significantly increased (by 13 %) after exposure to green laser irradiation. Another MTT assay was used for isolated mitochondria suspensions in order to examine the effect of green laser irradiation on stimulation of processes related to oxidative phosphorylation. It revealed 31.3 %-increase in MTT assay products in irradiated mitochondria as compared to controls. Laser irradiation of isolated mitochondria suspension did not significantly change 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy, indicating that mitochondrial membrane fluidity was not affected by laser light. Fluorescence emission spectra of irradiated as well as non-irradiated mitochondria suspensions showed fluorescence maximum at 635 nm, corresponding to emission of Protoporphyrin IX, which was significantly lower (by 20.7 %) in irradiated sample.

Key words: Mitochondria — Transmembrane potential — Oxidative phosphorylation — Membrane fluidity — Laser irradiation — Protoporphyrin IX

Introduction

The first publication about low-level laser therapy (LLLT) appeared more than 30 years ago (Kovács et al. 1974). Since then, the effectiveness and applicability of a variety of light sources, in the treatment of a wide range of medical conditions

Correspondence to: Peter Kaššák, Division of Biomedical Physics, Faculty of Mathematics, Physics and Informatics, Comenius University, Mlynská Dolina F1, 842 48 Bratislava 4, Slovakia. E-mail: pkassak@yahoo.com

has thoroughly been investigated, *in vitro* as well as *in vivo* (for reviews see Conlan et al. 1996).

In LLLT, the question is no longer whether light has biological effects but rather how radiation from therapeutic lasers and light emitting diodes (LEDs) works at the cellular and organism levels and what the optimal light parameters are for different uses of these light sources with various wavelengths (Karu 2003).

For the lower wavelength bands, there is lack of information about their effect on biological tissues and cells. Up today, green wavelengths band was not officially included into LLLT and stands on the edge of research interest in spite of its possible beneficial use. The fact that green light has small penetration depth (Šikurová et al. 2003) in the biological tissues does not have to be disadvantage as often mentioned, but also may be used in the very focused and gentle therapeutical interventions.

Wavelengths of 514 and 532 nm have been reported to change heart beating frequency, activation in contractibility and electrical activity in rat myocardial cells (Berns et al. 1972; Salet et al. 1979). Furthermore, using LED of wavelength 570 nm, Vinck et al. (2003) observed stimulation of the chicken fibroblast proliferation. Karu (1999, 2003) marginally reported in her works absorption of green band light by fibroblasts and small DNA synthesis activation. Increased activity of the membrane bound enzyme Na^+,K^+-ATPase after the irradiation by 532 nm laser light has been shown (author's data, not published).

Large number of studies (Conlan et al. 1996; El Batanouny et al. 2002; Webb and Dyson 2003) has been performed using red or infra-red lasers to observe biostimulating effect of these wavelengths on various tissues and cell related processes. Selected from those, Passarella et al. (1984) showed that irradiation of He-Ne laser (632.8 nm, 15 mW) generates an extra electrochemical potential and an increase in ATP synthesis within mitochondria. Later, Karu (2003) suggested that photoreceptors might be components of the respiratory chain. Also proposed that in wounds exhibiting delayed healing, the effect of low intensity visible light (632.8 and 760 nm) might stimulate cells to increase proliferation. Photoreception occurring at the mitochondrial level may intensify respiratory metabolism and the electrophysiological properties of the membrane, thus leading to changes in cell physiology.

In the present work we investigated green laser effect on B-14 cells and isolated rat heart mitochondria in the matter of altered redox state and changes in membrane properties as well as possible damaging photodynamic effect of the protoporphyrin IX (PpIX) – precursor of heme, located in the mitochondrial membrane and absorbing light in the range of our stimulation.

Materials and Methods

B-14 cells preparation and culture procedures

Cultures of B-14 (Chinese hamster ovarian) cells were maintained in culture medium (Dulbecco's modified Eagle's medium (DMEM) enriched by 10 % newborn calf serum and 1 % ampicilin/streptomycin). Cells were plated on 96-well plates

in density of 40,000 cells *per* well approximately 24 h before the experiment and stored at 37 ℃ in a humid atmosphere.

Heart mitochondria isolation

Isolation was performed on adult male Wistar rats weighing 290 ± 20 g fed with a standard Larsen diet and water ad libitum. The animals were sacrificed by decapitation in accordance with the Guide for the Care and Use of Laboratory Animals. Hearts were placed in an ice-cold isolation solution containing 180 mmol/l KCl, 4 mmol/l EDTA – Tris (pH 7.4) and cut into pieces. Isolation solution enriched by 0.1 % bovine serum albumin (BSA) was added together with protease (2.5 mg *per* g of heart tissue). Homogenisation with teflon pestle followed and heart mitochondria were isolated by differential centrifugation (Lehninger et al. 1967). Mitochondria obtained by this isolation procedure maintained mainly their inner membrane fully functional but were oriented inside out (Vrbjar et al. 1984; Ziegelhöffer et al. 2003; Ziegelhöffer-Mihalovičová et al. 2003; Waczulíková et al. 2004). All isolation procedures were carried out on ice.

Protein content in the isolated mitochondria was estimated by mean of the method described by Lowry et al. (1951) using BSA as standard. Pellet of isolated mitochondria was diluted by isolation solution to obtain concentration 0.25 g/ml of mitochondria.

Irradiation procedure

In our study, Nd:YAG laser (Raise Electronics, Taiwan) with output power 30 mW and emitting wavelength of 532 nm was used as a source of polarized and coherent light. Time of irradiation was set to 20 min obtaining light energy of 36 J. Each examined sample was irradiated alone receiving the radiant energy *per* unit area of the sample surface (fluence) of 1146 J/cm^2. Controls were maintained in dark and at the same conditions as irradiated samples. Irradiation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Sigma-Aldrich Inc., USA) solution itself did not show any changes or production of formazan crystals, respectively.

Trypan blue assay

Trypan blue dye exclusion assay is the most commonly used and accepted method for the measurement of cell viability. It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability. We added of 0.4 % (w/v) Trypan blue (Sigma-Aldrich Inc., USA) to cell suspension after irradiation procedure, mixed well, and scored under a phase contrast microscope (Li et al. 2003).

MTT assay

This assay is a quantitative colorimetric method to determine cell viability. It utilizes the yellow tetrazolium salt (MTT) which is metabolized by mitochondrial dehydrogenase enzyme from viable cells to yield a purple formazan reaction product

(Mosmann 1983). In our experiments, MTT assay was applied in cultured B-14 cells on wells as well as for rat heart mitochondria suspension in tubes.

On wells: culture medium was replaced with 50 μl of 0.2 mg/ml MTT solution in the incubation buffer (140 mmol/l NaCl, 5.4 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$, 0.9 mmol/l NaH_2PO_4, 3.8 g/l glucose, 1 mmol/l sodium pyruvate, 20 mmol/l HEPES, pH 7.4).

In tubes: mitochondria suspension was mixed with MTT yielding the final concentration of MTT 0.2 mg/ml and final concentration of mitochondria 12.5 g/l in the sample.

In both assays, incubation of cells loaded with MTT lasted two hours. Finally, solution over the attached or softly spun down cells was gently removed and produced formazan crystals were dissolved in 50 μl (in wells) and 200 μl (in tube) of DMSO respectively. Absorbance of dissolved formazan crystals was measured at 560 nm by using Stat Fax-2100 multiwell plate reader (Awareness Technology Inc., Florida, USA) and spectrometer Specord M40 (Carl Zeiss, Germany) respectively.

Mitochondrial transmembrane potential measurements

Changes in mitochondrial transmembrane potential of B-14 cells were monitored using the fluorescent probe 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; Molecular Probes Inc., USA). Probe solution in final concentration of 50 nmol/l was freshly prepared in incubation buffer before each measurement from 20 μmol/l $DiOC_6(3)$/DMSO stock solution. 50 μl of the probe solution was then added to an appropriate well. Fluoresecence intensity at 535 nm (excitation at 485 nm) was measured on a Fluoroskan Ascent FL (Labsystems, Finland) multiwell fluorescence plate reader.

Membrane fluidity measurements

Steady-state fluorescence anisotropy (r) of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma-Aldrich, Inc., USA) was evaluated as an indicator of structural ordering in the hydrophobic core of the membrane, which is in inverse proportion to membrane fluidity. The stock solution of DPH (5×10^{-4} mol/l in acetone) was further diluted with Tris-KCl-EDTA solution (pH 7.4) to concentration of 2×10^{-6} mol/l. Mitochondria suspensions were then stained with the DPH probe by 1 : 1 dilution yielding the final DPH concentration of 1×10^{-6} mol/l and the final concentration of mitochondria of 3.125 g/l. Steady-state fluorescence anisotropy values were obtained from the Perkin-Elmer LS 45 fluorimeter, based on measurements of I_{VV} and I_{VH} i.e., the fluorescence intensities polarized parallel (V) and perpendicular (H) to the vertical plane of polarization of the excitation beam, respectively. The fluorescence anisotropy is defined by the equation

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where G equals I_{HV}/I_{HH} and is the correction factor for instrumental artefacts.

Simultaneously, fluorescence emission spectra of suspensions of isolated mitochondria were recorded at the wavelength range 515–700 nm (excited at 488 nm) to monitor the presence and characteristics of PpIX after irradiation.

If not stated elsewhere, all experiments were performed at room temperature (22 ± 2 ℃).

Statistical analysis

Groups of data were checked for normal distribution by Shapiro–Wilk test. The experiments were carried out in pair experiments and there for paired t-test was used for parametrical analysis. Statistical significance for all tests was accepted at the 0.05 level and lower.

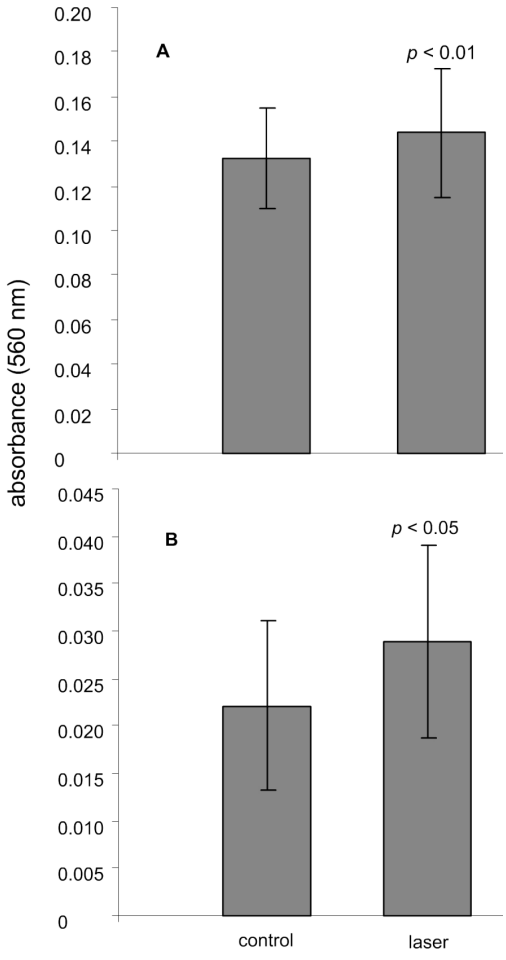

Figure 1. Absorbance (at 560 nm) of MTT assay products in non-irradiated (control) and irradiated (laser) B-14 cells (A) and isolated rat heart mitochondria (B). Column heights represent mean ± S.D. ($n = 18$ for A and $n = 7$ for B)

Results

To ensure that laser irradiation (532 nm, 30 mW, 1146 J/cm^2) itself does not kill the cells, survival of B-14 cells after laser irradiation was examined using Trypan blue dye exclusion assay. There was no significant difference in the amount of dead cells in non-irradiated and irradiated sample (data not shown).

Metabolic activity of B-14 cells after irradiation was estimated by MTT assay. We found that laser illumination resulted in a significantly ($p = 0.003$) higher absorbance values (by 8 %) of dissolved purple formazan crystals in irradiated samples in comparison to non-irradiated controls (Fig. 1A).

Another step was to examine mitochondrial transmembrane potential of B-14 cells after exposure to green laser irradiation by means of the $DiOC_6(3)$ fluorescent probe. Laser irradiation of B-14 cells resulted in a statistically significant ($p = 0.017$) increase (by 13 %) in $DiOC_6(3)$ fluorescence intensity in comparison to controls (Fig. 2).

MTT assay was also used in isolated mitochondria suspension to examine the effect of green laser irradiation on stimulation of processes related to oxidative phosphorylation. Production of formazan crystals from the MTT measured by absorbance at 560 nm was significantly ($p = 0.033$) higher (by 30.3 %) in irradiated mitochondria compared to controls (Fig. 1B).

Changes in mitochondrial membrane fluidity after irradiation were assessed in terms of DPH fluorescence anisotropy. Kinetics of DPH incorporation was monitored during 30 min following the addition of DPH to mitochondria suspension

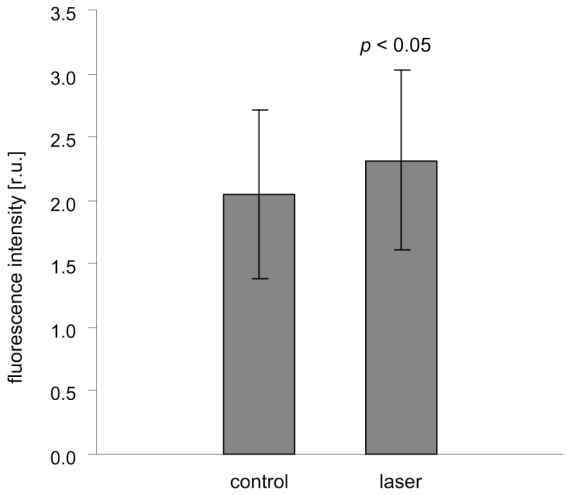

Figure 2. Transmembrane potential of B-14 cells evaluated by fluorescence intensity of $DiOC_6(3)$ (emission at 535 nm; excitation at 485 nm) in non-irradiated (control) and irradiated (laser) samples. Values are shown as mean ± S.D. of 6 independent experiments.

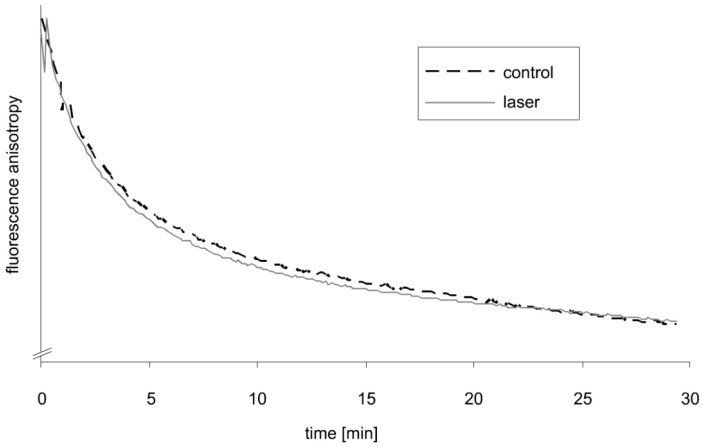

Figure 3. Time course of fluorescence anisotropy of DPH in the control, non-irradiated mitochondria (dashed black line), and in mitochondria irradiated by green laser light (solid grey line). The data presented are mean values of ten independent experiments; S.E.M. did not exceed 6%. The minimum changes are not statistically significant.

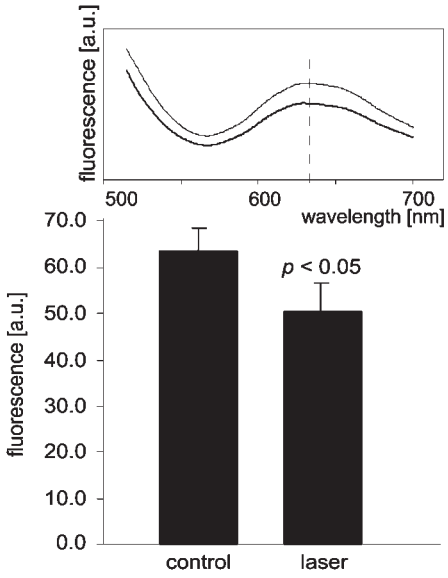

Figure 4. Laser light-induced changes in intensity of the 635 nm fluorescence peak (excitation at 488 nm) of isolated rat heart mitochondria. Data are shown as mean ± S.D. of 5 individual measurements. Insert: fluorescence emission spectrum (excitation at 488 nm) of isolated rat heart mitochondria suspension in case of an irradiated (black line) and non-irradiated (grey line) sample.

for irradiated as well as non-irradiated samples (Fig. 3). As observed, laser irradiation of isolated mitochondria suspension does not significantly change DPH fluorescence anisotropy, indicating that mitochondrial membrane fluidity was not affected by laser light.

Concurrent with fluidity measurements, fluorescence emission spectra of irradiated and non-irradiated mitochondria suspensions were recorded to establish the fluorescence of PpIX and its possible changes. A representative example of fluorescence spectra of isolated rat heart mitochondria suspension, when illuminated or kept in the dark, are presented in Fig. 4. Both spectra show fluorescence maximum at 635 nm, corresponding to emission of PpIX. Laser irradiation of isolated mitochondria did not result in a shift of the peak position or spectral shape. The only differences were in the values of fluorescence intensity, which showed a significant decrease (by 20.7 %, $p = 0.022$) in irradiated sample.

Discussion

Laser induced changes in the biological tissues requires the absorption of the laser light energy by a specific molecule. Only then the energy of laser irradiation may be used as stimulus for reactions. The primary mechanism of light action after absorption of light quanta and the promotion of electrically excited states have not been established. Possible explanations include stimulation of ascorbic acid uptake by cells, stimulation of photoreceptors in the mitochondrial respiratory chain, changes in cellular ATP or AMP levels, and cell membrane stabilization (Conlan et al. 1996).

In mitochondria, five possible mechanisms have been discussed in previous studies (El Batanouny et al. 2002; Karu 2003) including singlet-oxygen hypothesis, redox properties alteration hypothesis, nitric oxide hypothesis, superoxide anion hypothesis and transient local heating hypothesis. However, these were postulated for red and infra-red light operating lasers. Green laser light was not included, but we may apply some of these hypotheses to explain or discuss our results.

In present work we showed that the irradiation by green laser significantly increases (by 8 %) the ability of the B-14 cells to reduce tetrazolium salt (MTT) to formazan crystals (Fig. 1A). Reduction of MTT in isolated cells and tissues is regarded as an indicator of cell redox activity. The reaction is attributed mainly to mitochondrial enzymes and electron carriers (Bernas and Dobrucki 2002). Thus we may suggest that the increase in the MTT degradation is closely related to the positive stimulation of the mitochondrial enzymes and electron carriers by green laser light. Suggestion is consistent with the results on mitochondrial transmembrane potential of B-14 cells monitored by the $DiOC_6(3)$ fluorescent probe as well as on MTT assay carried out on isolated rat heart mitochondria.

We observed the significant increase in $DiOC_6(3)$ fluorescence intensity in irradiated samples of B-14 cells (Fig. 2), which could be attributed to the increase in mitochondrial transmembrane potential, as $DiOC_6(3)$ fluorescence intensity is increased by the H^+ exchange between external glutamate and internal aspartate

or cysteinsulfinate (Stipani et al. 1983). The mitochondrial transmembrane potential is a sensitive indicator for the energetic state of the mitochondria and therefore the whole cells (Brand et al. 1994), and can be used to assess the activity of the mitochondrial proton pumps, electrogenic transport system, and the activation of the mitochondrial permeability transition (Zoratti and Szabo 1995). It is known that mitochondria respiratory chain pumps protons outwards and therefore it creates positive transmembrane mitochondrial potential outside the inner membrane.

Although inhibition of oxidative phosphorylation in the presence of $DiOC_6(3)$ has been reported (Rottenberg and Wu 1998), according to the literature, inhibitory effect is not significant in the concentration used.

As we suggested above that observed effect of laser on whole cells is closely related to mitochondria, in next experiments we focus on investigation of laser effect on isolated mitochondria. Irradiated isolated rat heart mitochondria degraded MTT to formazan crystals by 30.3 % in comparison to non-irradiated controls (Fig. 1B). This increase is dramatic in comparison to slight 8 % stimulation in B-14 cells and was probably caused by the free exposure of the isolated material to the laser radiation. The redox properties alteration hypothesis postulates that photoexcitation of certain chromophores in the cytochrome c oxidase molecule (like Cu_A and Cu_B or hemes a and a_3) influences the redox state of these centres and, consequently, the rate of electron flow in the molecule (Karu 2003). MTT degradation in the mitochondria is performed by various enzymes in there, mainly by enzymes related to oxidative phosphorylation.

The supply of energy by the mitochondrion depends on the maintenance of the chemiosmotic gradient across its inner membrane (Mitchell 1979). This gradient, also known as the proton motive force, is generated by three respiratory enzyme complexes which use the free energy released during electron transport to translocate protons from the mitochondrial matrix into the intermembrane space. Proton motive force has two components: the mitochondrial membrane potential (negative in matrix) and the pH gradient (alkaline in matrix) (Mathur et al. 2000). Changes in mitochondrial transmembrane potential are integral to cell life. In normal cell function, the maintenance of transmembrane potential is essential for ATP synthesis (Škárka and Ošťádal 2002).

As we presented, laser irradiation does not affect significantly the degree of DPH fluorescent anisotropy (Fig. 3), which corresponds to unchanged ordering of phospholipid molecules in the hydrocarbon region of membranes (lipid packing), i.e. to unchanged mitochondrial membrane fluidity. However, we observed changes in PpIX fluorescence intensity (Fig. 4). The prosthetic group of heme in cytochromes b, c_1 and c is iron PpIX, the same heme as in myoglobin and hemoglobin (Berg et al. 2002), which are reported to have high absorption of green light. PpIX is also synthesized from 5-aminoleavulinic acid (5-ALA) in the mitochondria and then diffuses into the cytoplasm of the cell (Peng et al. 1997; Tabata et al. 1997; Zhang et al. 2000). After absorption of laser light energy by PpIX, photobleaching could occur due to its photodynamic action. However, the concentration of the PpIX in isolated mitochondria is suggested to be very low, and thus the photodynamic effect has

limited affection on membrane structure, and does not cause significant dysfunction of the membrane and membrane bonded processes. Production of reactive oxygen species has been reported (Oleinick et al. 2002) to cause the dissipation or collapse of the mitochondrial transmembrane potential and decrease in the membrane fluidity (Ricchelli et al. 2001), what is not in agreement with our results. We have found out insignificant changes of membrane fluidity but significant increase in the mitochondrial transmembrane potential. These results does not support occurrence of damaging photodynamic effect of PpIX in presence of laser irradiation.

All our experiments were performed at room temperature, which is below the optimal temperature for living cells. We may only suppose that the observed effect would be more notable in the proper temperature conditions.

In conclusion we can say that our experimental results proved that mitochondrial functional alterations occur in response to 532 nm low level laser irradiation. Of these, in particular, the increase in mitochondrial oxidative phosphorylation processes may indicate a biostimulating effect. However, further experiments exploring applications of LLLT should follow.

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