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ORIGINAL ARTICLE

Are the mitochondrial respiratory complexes blocked by NO the targets for the laser and LED therapy?

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Abstract Effects of laser (442 and 532 nm) and light-emitting diode (LED) (650 nm) radiation on mitochondrial respiration and mitochondrial electron transport rate (complexes II–III and IV) in the presence of nitric oxide (NO) were investigated. It was found that nitric oxide (300 nM–10 μM) suppresses mitochondrial respiration. Laser irradiation of mitochondria (442 nm, 3 J cm^{-2}) partly restored mitochondrial respiration (approximately by 70 %). Irradiation with green laser (532 nm) or red LED (650 nm) in the same dose had no reliable effect. Evaluation of mitochondrial electron transport rate in complexes II–III and IV and effects of nitric oxide demonstrated almost similar sensitivity of complex II–III and IV to NO, with approximately 50 % inhibition at NO concentration of 3 μM. Subsequent laser or LED irradiation (3 J cm^{-2}) showed partial recovery of electron transport only in complex IV and only under irradiation with *blue* light (442 nm). Our results support the hypothesis of the crucial role of cytochrome *c* oxidase (complex IV) in photoreactivation of mitochondrial respiration suppressed by NO.

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Introduction

In spite of a great variety of clinical applications of low-level laser therapy [1–5], the mechanism of beneficial effects of low-power laser and light-emitting diode (LED) on human and animal cells and tissues is still a matter of discussion. The central point of the problem seems to be, which molecular targets for photons of visible and near infrared light are responsible for beneficial systemic effects.

Four primary action mechanisms were reviewed by T. Karu: (1) changes in the redox properties of the respiratory chain components following photoexcitation of their electronic states, (2) generation of singlet oxygen, (3) localized transient heating of absorbing chromophores, and (4) increased superoxide anion production [6]. The analysis of experimental data accumulated until 1994 allows as to propose three new hypotheses about the molecular mechanism of action of low-power laser light [7]: (1) photodynamic action on membranes accompanied by intracellular calcium increase and cell stimulation, (2) photoreactivation of Cu and Zn superoxide dismutase, and (3) photolysis of the hemeprotein nitrosyl complexes with free nitric oxide (NO) release. It was postulated that these three effects underlie the indirect bactericidal, regenerative, and vasodilatation action of laser radiation. The experimental data corroborating this hypothesis were reviewed in [8].

An alternative mechanism of the therapeutic effect of low-level laser radiation, based on the photodissociation of hemeprotein nitrosyl complexes, was substantiated in experiments with model systems, such as NO-hemoglobin [9, 10] and NO-cytochrome *c* [11, 12].

The photosensitivity of heme and non-heme nitrosyl complexes in the mitochondrial respiratory chain is of particular interest, as these complexes are formed in vivo and may limit the rate of oxygen consumption [13–16]. That is why nitrosyl complex photolysis may essentially contribute to the therapeutic effects of low-level laser and LED radiation. The role of cytochrome *c* oxidase as a light acceptor at certain circumstances was a matter of many investigations (see review [17]). The virtual photosensitivity of cytochrome *c* oxidase was shown in some investigations, but the NO complex restored very quickly so that the photochemical effect could be observed either within a millisecond time scale or at very low temperature (in the 30–70 K region) [18]. So, it remains unclear whether the NO complex of cytochrome *c* oxidase can be a target for a therapeutic dose of laser and LED in natural conditions or not.

The main goal of the present study was to elucidate the major mechanisms of low-level laser and LED therapy effects on mitochondrial respiration and mitochondrial electron transport in the presence of nitric oxide. To pinpoint the localization of mitochondrial centers sensitive to laser and LED radiation, we evaluated the electron transport rate in II–III and IV green complexes of the mitochondrial respiratory chain. The rate of mitochondrial electron transport by II–III complexes (succinate dehydrogenase–cytochrome bc_1) was evaluated by the rate of cytochrome *c* reduction by the electron transport chain in the presence of succinate as the substrate of oxidation. To estimate the mitochondrial complex IV activity (cytochrome *c* oxidase), we measured the oxidation rate of ferrocytochrome *c*, added to mitochondria with disrupted outer membrane integrity.

Materials and methods

Mitochondrial isolation

Rat liver mitochondria were isolated from 2–3-month-old male albino rats (300±30 g) using standard protocol described in [19]. For mitochondrial isolation, we used extraction and storage buffers. The extraction buffer (pH 7.45) contained 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA. The storage buffer (pH 7.45) contained 10 mM HEPES, 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, and 2 mM K_2HPO_4. Mitochondria were quantified by protein content evaluated by biuret assay in the presence of bovine serum albumin (BSA) as a standard. All manipulations with animals were performed in accordance with the ethical guidelines established by the Institutional Animal Care and Use Committee.

Oxygen consumption measurement

Mitochondrial oxygen consumption measurements were carried out electrochemically by means of a membrane-covered platinum electrode at 25 °C (Clark-type electrode, Econix-Expert, Russia) in the presence of sodium succinate (10 mM) and ADP (0.2 mM) as substrates. All solutions were prepared using Millipore purified water (18.2 M Ω cm^{-1}). Each measurement was made at least in triplicate. Linear approximation of the experimental points was used to properly calculate the mitochondrial respiration rates V_3 and V_4. The time period used for oxygen consumption measurements was 100 s.

Laser irradiation of samples

He–Cd laser (λ=442 nm, output power of 20 mW) GKKL-20B (Plasma, Russia), diode-pumped solid-state laser (λ=532 nm, output power of 20 mW) LCS-DTL-317 (Laserexport, Russia), and LED (λ=650 nm, output power of 40 mW) KLM-H650 (FTI-Optronic, Russia) were used as a sources of radiation. The laser and LED light sources had continuous beam intensity. The equipment used for the irradiation of the samples was thoroughly calibrated. To evaluate the irradiation power, we used Optical Power Meter 1916-C (Newport, USA). The results of calibration completely matched the information given by the manufacturer. This information and other parameters of the radiation sources are presented in Table 1. Irradiation of the samples was continuous (not pulsed) to obtain the desired dose. The mitochondrial suspension was moved into a 2-ml Eppendorf tube containing mitochondrial suspension and then placed into the stirrer vertically with the lid open. The source of radiation was located at a distance of 20 cm above the Eppendorf tube, with the light aperture turned down. The diameter of the laser beam was 5 mm at the surface of the mitochondrial suspension. The Eppendorf tube was spinning around the light beam as an axis, to have the entire surface illuminated evenly. The sample was irradiated with permanent solution stirring.

Table 1 Laser and LED irradiation parameters. A continuous laser beam was used

Parameter	He–Cd laser	Diode-pumped solid-state laser	LED
Wavelength, nm	442	532	650±5
Power, mW	20±2	20±2	40±5
Irradiation time, s	30–300	30–300	15–150
Beam area, mm^2	15.7	15.7	15.7
Total energy, J cm^{-2}	3–31	3–31	3–31
Power density, W cm^{-2}	0.03±0.01	0.03±0.01	0.06±0.01

Complex II–III activity measurement

The activity of mitochondrial complexes II–III was measured in mitochondria with disrupted outer membrane integrity for better cytochrome *c* access to the complex reactive site (as well as in the measurements of the cytochrome *c* oxidase activity) [19]. Mitochondrial suspension (protein content 100 μg ml^{-1}) in Tris–HCl buffer (10 mM Tris–HCl and 125 mM KCl, pH 7.0), containing 1-mM sodium succinate and 1-mM NaN_3, was placed into the cuvette with optical path 1 cm. The suspension was gently shaken, and optical density kinetics was monitored at 550 nm from the 5th to the 45th second with a 5-s time interval. The difference in extinction coefficients of the reduced and oxidized cytochrome *c* forms was taken as $\Delta\varepsilon^{550}$=21.84 mM^{-1} cm^{-1} [20]. Total complexes II–III activity ($A_{II–III}$) was calculated according to the equation:

$$A_{\text{II–III}}(\text{un./mg protein}) = \frac{\Delta A/\text{min} \times 585}{\Delta\varepsilon^{550} \times 50},$$

where ΔA/min is the rate of optical density growth from the 5th to the 45th second at 550 nm, $\Delta\varepsilon^{550}$ is the cytochrome *c* molar extinction coefficient at 550 nm, and 585 and 50 are the final sample volume and mitochondrial suspension volume, correspondingly.

Complex VI (cytochrome *c* oxidase) activity measurement

Cytochrome *c* oxidase activity was evaluated in mitochondria with disrupted outer membrane integrity for better cytochrome *c* access according to [19]. Cytochrome *c* (0.225 mM) was reduced by addition of 5 mM dithiothreitol. Mitochondrial suspension (protein content 40 μg ml^{-1}) and reduced cytochrome *c* solution (11.25 μM) in Tris–HCl buffer (10 mM Tris–HCl and 125 mM KCl, pH 7.0) were placed into the cuvette with optical path 1 cm. The suspension was gently shaken and optical density kinetics was monitored at 550 nm from the 5th to the 45th second with a 5-s time interval. The difference in extinction coefficients of the reduced and oxidized cytochrome *c* was taken as $\Delta\varepsilon^{550}$=21.84 mM^{-1} cm^{-1} [20]. Complex IV activity (A_{IV}) was calculated according to the equation:

$$A_{\text{IV}}(\text{un./mg protein}) = \frac{\Delta A/\text{min} \times 555}{\Delta\varepsilon^{550} \times 20},$$

where ΔA_{min} is the rate of optical density decay from the 5th to the 45th second at 550 nm, $\Delta\varepsilon^{550}$ is the cytochrome *c* molar extinction coefficient at 550 nm, and 555 and 20 are the sample final volume and mitochondrial suspension volume, correspondingly.

Statistics

Each value in the experimental data is presented as the mean± SEM of at least three independent measurements. To compare the mean values, modified Student's *t* test was used.

Results

As it was mentioned above, heme- and non-heme mitochondrial nitrosyl complexes are recognized to be targets for laser and LED radiation [21–23]. The formation of these complexes may occur in reactions of nitric oxide with hemoproteins or iron–sulfur clusters in mitochondria, whenever NO is produced in the organelles or enters from the outside. These reactions would decrease the oxygen consumption rate. In the first series of experiments, we investigated the relationship between the amount of nitric oxide added and mitochondrial respiration rate.

We have found that the addition of NO to mitochondria (0.3–10 μM) leads to a gradual decrease of the mitochondrial respiration rate in metabolic states 3 and 4 (V_3 and V_4) and of the respiratory control ratio (RCR) (Fig. 1). It should be mentioned that the V_3 rate and RCR dropped down already at 0.3 μM NO, while the V_4 rate started to go down at NO concentration of 2 μM and more. Finally, V_3 reached the zero level and RCR minimal possible value (one) at NO concentration of 10 μM.

In further experiments, we used the concentration of NO equal to 0.3 μM, at which V_3 dropped by 50 % while V_4 did not change at all. Note that this concentration of NO is close to the physiological level (0.2 μM [16]).

An investigation of laser and LED effects on mitochondria subjected to NO demonstrated a statistically significant rise of the V_3 rate (approximately 60 %) and almost no changes in the V_4 rate under irradiation with laser light at 442 nm. In the case of irradiation at 532 and 650 nm, no changes in the V_3 or V_4 rates were detected (Fig. 2).

It was shown in numerous publications that NO-hemoproteins and iron–sulfur nitrosyl complexes are photosensitive and would release free nitric oxide upon irradiation [24, 25, 11, 26]. Nevertheless, there is yet no information on what kind of centers in the mitochondrial electron transport chain are responsible for the interaction with nitric oxide and sensitive to the subsequent laser and LED radiation. To clarify this problem, we have studied the effects of physiological concentrations of NO on the rate of mitochondrial electron transport by respiratory complexes II–III and IV, and the action of laser and LED irradiation on these complexes in the absence or presence of NO. The rate of electron transport in complexes II–III (succinate dehydrogenase–cytochrome bc_1) was evaluated by means of the cytochrome *c* reduction.

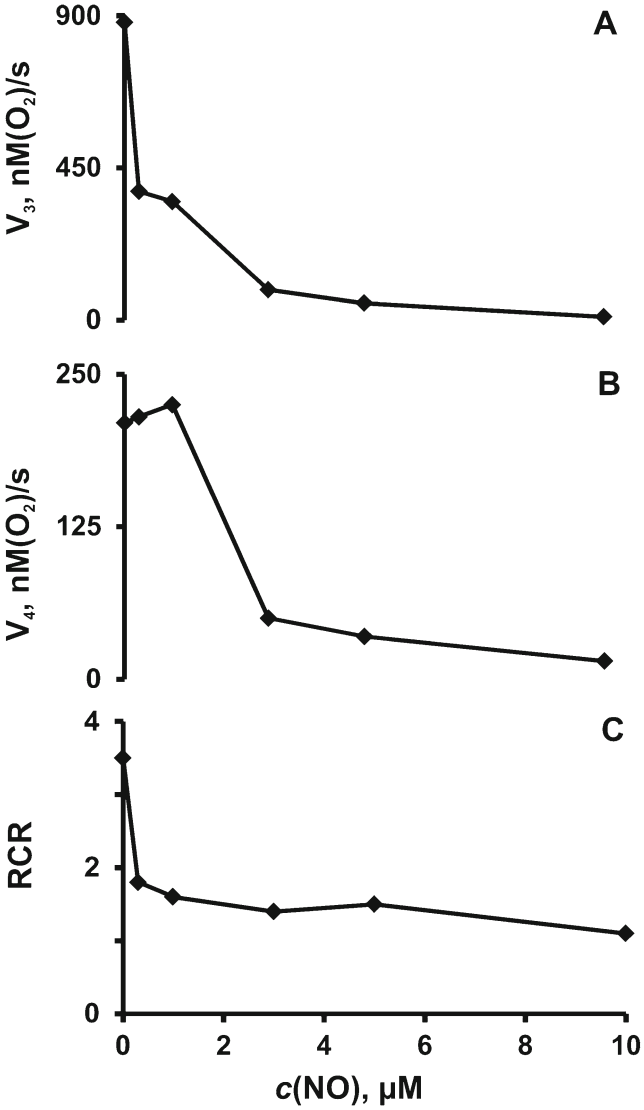

Fig. 1 Effects of NO on mitochondrial oxygen consumption in metabolic state 3 (V_3) (**a**), state 4 (V_4) (**b**), and respiratory control ratio (RCR) (**c**). Mitochondrial concentration 1 mg ml^{-1}. Storage buffer (pH 7.45) in the presence of sodium succinate (10 mM) and ADP (0.2 mM) as substrates (n=3)

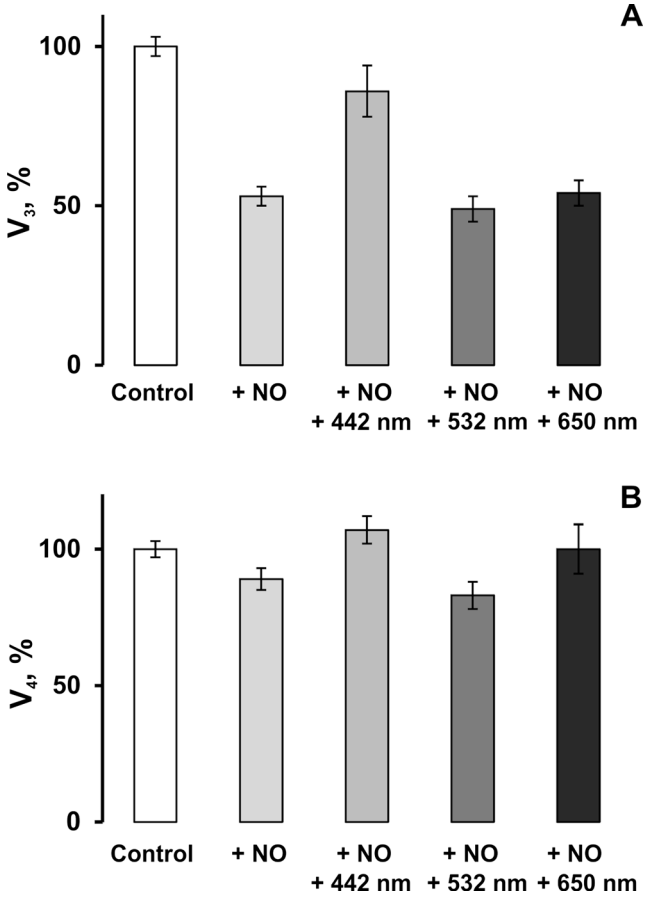

Fig. 2 Effects of laser (442 and 532 nm) and LED (650 nm) on oxygen consumption rates of mitochondria, subjected to nitric oxide, in metabolic state 3 (V_3) (**a**) and state 4 (V_4) (**b**). Irradiation dose 3 J cm^{-2}, NO 0.3 μM, Na succinate 10 mM, and ADP 0.2 mM. V_3 and V_4 are presented as a percent to the control (n=3)

As a source of reducing equivalents, sodium succinate was used, a specific substrate for complex II. Complex IV (cytochrome *c* oxidase) was completely blocked in these experiments by NaN_3, in order to prevent the cytochrome *c* reoxidation.

Figure 3a shows a statistically significant change in electron transport in complexes II–III only when mitochondria were irradiated with blue laser light (442 nm). On average, the complex II–III activity decreased by 30 % and more at all irradiation doses used. No changes were detected in the rate of electron transport in complexes II–III when laser (532 nm) or LED (650 nm) irradiation was applied. Effects of laser (442 and 532 nm) and LED (650 nm) irradiation on complex IV activity are presented in Fig. 3b. The electron transport rate in this case was practically unchanged at any dose and any wavelength of irradiation.

Panels a and b of Fig. 4 show the effects of NO on mitochondrial electron transport in complexes II–III and IV. NO in the concentration range 1–10 μM inhibited gradually the electron transport through complexes II–III and IV in mitochondria. At the concentration 3 μM, the electron flow decreased by roughly 50 %. This concentration of NO was used in the following experiments on the action of laser and LED radiation on II–III and IV complexes preincubated with NO.

The results of these experiments are presented in Fig. 5. One can see that blue laser irradiation (442 nm, 3 J cm^{-2}) decreased the activity of the II–III complexes inhibited by NO additionally by 14 % (totally by 72 %). Effects of green laser (532 nm, 3 J cm^{-2}) and red LED (650 nm, 3 J cm^{-2}) radiation were practically negligible (Fig. 5a). In contrast, the activity of complex IV inhibited by NO *increased* by 23 % (to total activity of 78 % as compared to the control). Irradiation of complex IV with laser light 532 nm or LED light 650 nm did not change the complex IV activity (Fig. 5b).

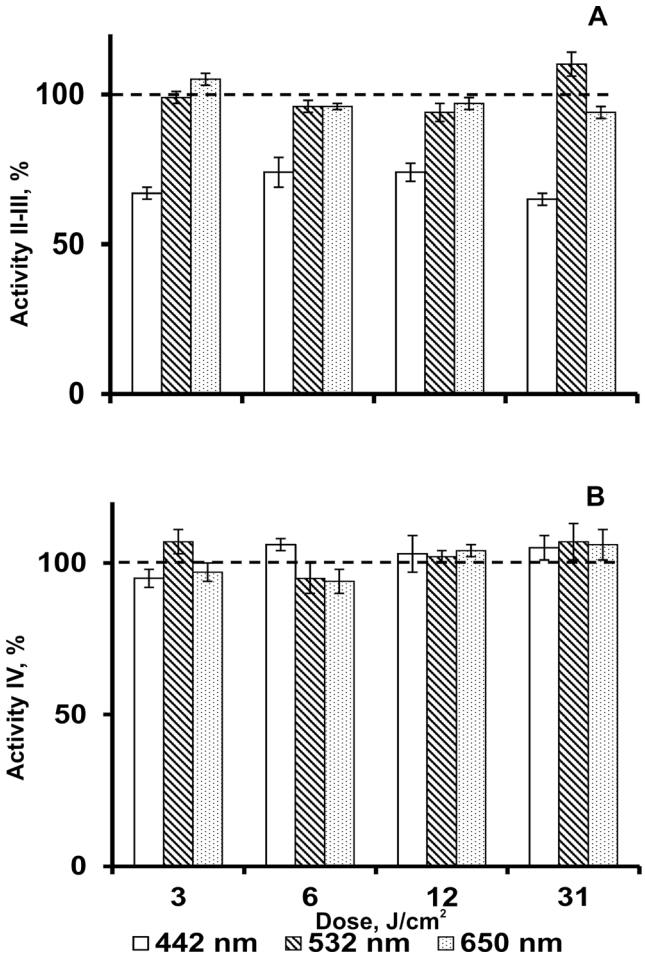

Fig. 3 Effects of laser (442 or 532 nm) and LED (650 nm) radiation on complexes II–III (**a**) and complex IV (**b**) activity. Mitochondrial concentration 1 mg ml^{-1} in Tris–HCl buffer 10 mM in the presence of 125 mM KCl, pH 7.0

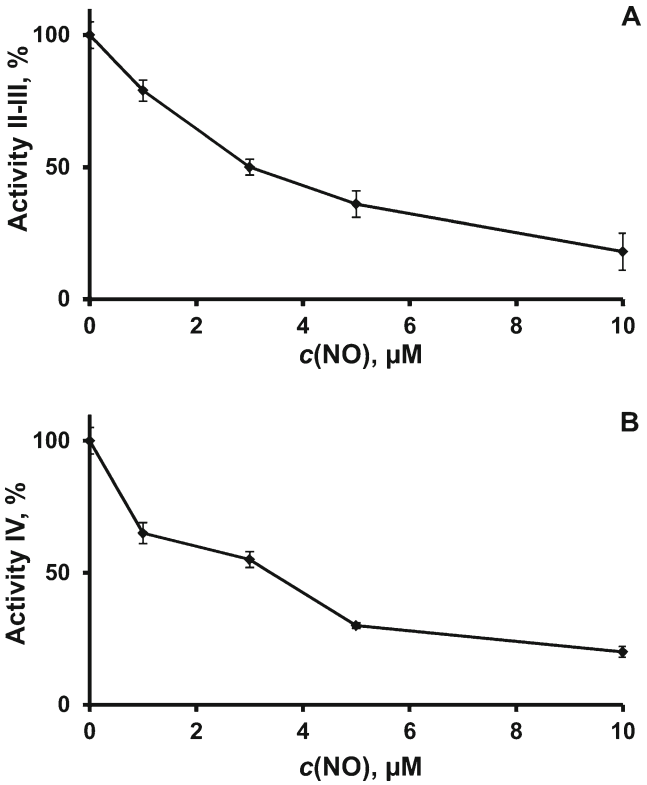

Fig. 4 Effect of NO on complexes II–III (**a**) and complex IV (**b**) activity. Mitochondrial concentration 1 mg ml^{-1} in Tris–HCl buffer 10 mM in the presence of 125 mM KCl, pH 7.0

Discussion

The therapeutic effects of laser and LED radiation are well documented [27–29]. Apparently, the mitochondrial electron transport chain is the main target for the laser and LED radiation [30, 31]. Morimoto [32] and Yu [31] showed that irradiation of isolated mitochondria in metabolic state 3 with laser light of 514.5 or 660 nm enhances respiration, but irradiation with 351 or 458 nm inhibits it. The damaging effects of blue radiation on mitochondria in living cells and apoptosis development were observed after laser irradiation at 488 nm [33, 34]. The photochemical and photobiological processes responsible for these reactions are still a matter of discussion.

The damaging effects of laser radiation were demonstrated in our preceding publication [23], where the mitochondrial respiration rate in metabolic state 4 (respiratory control) increased upon irradiation with laser light (442 or 532 nm) or that of LED (650 nm) in the doses of 3 J cm^{-2} or higher. This fact suggests the disturbance of the inner mitochondrial membrane barrier properties under strong illumination. In parallel, we observed the decrease of the mitochondrial respiration rate in metabolic state 3 (phosphorylating state) upon laser (532 nm) or LED (650 nm) irradiation at the dose 3 J cm^{-2}.

In the present paper, we attempted to investigate the laser and LED effects on the electron transport rate in complexes II–III and IV. It was found that doses of 3–31 J cm^{-2} do not show any effect on the electron transport rate in complex IV, if mitochondria are irradiated at 442, 532, or 650 nm. On the other hand, the electron transport rate in mitochondrial complexes II–III had a 30 % fall after illumination by blue laser light (442 nm). Neither green (532 nm) nor red (650 nm) visible radiation produced a pronounced effect on respiration rate V_3 (Fig. 3). These observations show that the light-sensitive area is located within complexes II–III and that the absorption band is closer to 442 nm than to 532 or 650 nm. It is also worthwhile that the degree of the respiration inhibition was the same in the range of irradiation doses between 3 and 31 J cm^{-2}, showing that the complete photolysis of the photosensitive site does not stop the electron transport completely, but only restricts its rate. This phenomenon is probably responsible for mitochondrial membrane damage described in [32]. In the latter paper, it was shown that intensive violet–blue light stimulated H_2O_2 production by peroxisomes and mitochondria in cultured mouse, monkey, and human cells

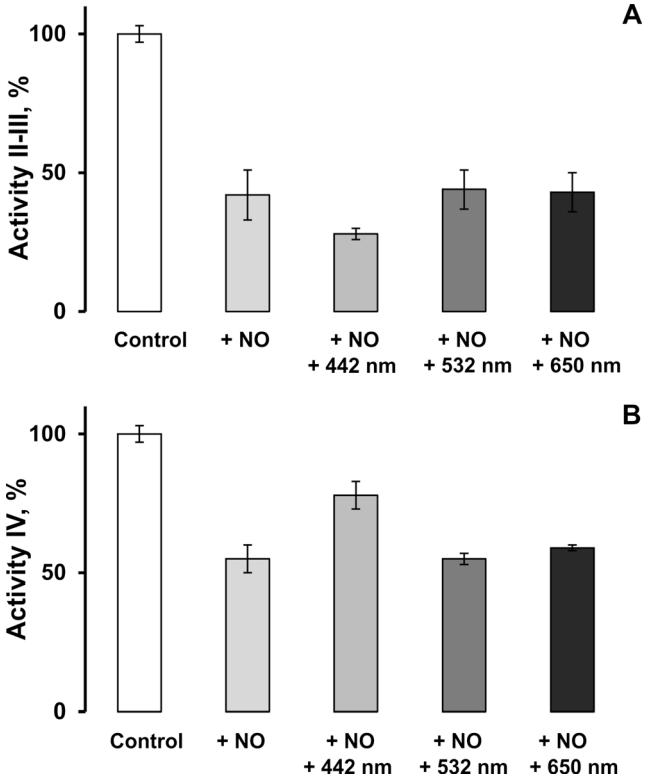

Fig. 5 Effects of laser (442 or 532 nm) and LED (650 nm) radiation on complexes II–III (**a**) and complex IV (**b**) activity in the presence of NO. Irradiation dose 3 J cm^{-2}, NO 3 μM in Tris–HCl buffer 10 mM in the presence of 125 mM KCl, pH 7.0

and that it was enhanced in cells overexpressing flavin-containing oxidases. This mechanism might be responsible for the depression of electron transfer in complexes II–III after illumination of mitochondria by 442-nm laser light, observed in our experiments (Fig. 3a).

It was found in our previous communication that the oxygen consumption by mitochondria in metabolic state 3 increased dramatically upon irradiation with blue laser light (442 nm) at doses 3–12 J cm^{-2} [23]. Interestingly, the activating laser effect was more pronounced (30±5 % of control) in mitochondria isolated from rats with experimental endotoxic shock, as compared to intact animals (12±5 % of control). Having in mind that the injection of lipopolysaccharide is known to produce strong activation of phagocyte inducible NO-synthase, increased synthesis of nitric oxide and, finally, the formation of nitrosyl complexes [35–37], these data may be explained if we propose that the isolated mitochondria contain some amount of NO (higher under endotoxic shock) that blocks the respiratory chain electron transport. The subsequent laser or LED illumination would remove NO from nitrosyl complexes and in this way increase the mitochondrial respiration rate.

The data obtained in the present paper support this assumption. It was shown that NO (0.3 μM) induces a twofold decrease of mitochondrial respiration rate in metabolic state 3 (without any effect on the outer mitochondrial membrane integrity-dependent metabolic state 4; see Fig. 1). Mitochondria irradiation (3 J cm^{-2}) with laser 442 nm restored respiration up to 86±8 % of the initial value (Fig. 2a). Green (532 nm) laser and red (650 nm) LED light did not show any effect on mitochondrial respiration rate in metabolic state 3 (Fig. 2a).

The rate of electron transport in mitochondrial electron transport chain complexes II–III (from succinate to cytochrome *c*) and complex IV (from cytochrome *c* to oxygen) was studied in the present paper too. It was shown that the inhibition of electron transport by nitric oxide and its derivatives, produced upon addition of 3 mM NO to mitochondria, was similar in all complexes studied (see Fig. 4a, b). Subsequent irradiation of these complexes at any wavelength (442, 532, or 650 nm; 3 J cm^{-2}) gave no increase in complex II–III activity. Moreover, when complexes II–III were irradiated with blue laser (442 nm), a decrease in complex II–III activity was observed (Fig. 5a). The cause of the complex II–III activity decrease could be the production of the nitrosyl complexes of cytochromes c_1, b_H, or b_L in complex III, iron–sulfur clusters in complexes II and III, or cytochrome *c*. *Principally*, all these NO complexes possess photosensitivity and must undergo decomposition upon irradiation leading to the restoration of complex activity [12, 8, 11]. The fact that we did not see this phenomenon means that complexes' activity inhibition could be due to the effects not of NO but of its derivatives. In particular, NO inhibition in complex II–III activity can be a result of peroxynitrite ($ONOO^-$) production rather than that of nitrosyl complexes [16].

In contrast to green and red light, irradiation with blue (442 nm) laser restored complex IV activity, suppressed by NO (Fig. 5b). We can suppose that the decrease of complex IV activity was due to the interaction of NO with cytochrome *c* oxidase and restoration of the complex IV activity is a result of the photolysis of nitrosyl complexes [38].

$$[\text{ox}-\text{Cyt}C] + \text{NO} \rightarrow [\text{ox}-\text{Cyt}C]-\text{NO} \xrightarrow{\text{photon}} [\text{ox}-\text{Cyt}C] + \text{NO}$$

The photoreactivation of CytOX by blue laser light (Fig. 5b) increases oxygen consumption by mitochondria suppressed by NO (Fig. 2a).

These experimental facts seem to be of practical interest to physicians exercising laser therapy. A question that should be answered is why blue light (442 nm) was much more effective than green (532 nm) or red (650 nm) light. One of the possible answers is that the absorbency of hemoproteins, such as cytochromes *a* and a_3, is one order of magnitude higher in the violet–blue region (near 442 nm) than in green (532 nm) and two orders of magnitude higher than in the red band (650 nm) [39]. In the publications by T. Karu, action spectra of laser radiation in the visible wavelength range (405, 620, 680, 760, and 820 nm) on cell synthesis of nucleic acids and

cell adhesion were studied [40–42]. It was found that effects of blue and red light were comparable only when the dose of blue light was one or two orders of magnitude greater than the dose of red or IR radiation. This experimental finding corresponds to our hypothesis on the pivotal role of cytochrome *c* oxidase and its nitrosyl complexes in the effects of laser radiation on mitochondria.

Conclusions

The effects of nitric oxide and laser and LED radiation on mitochondrial complexes II–III and IV have been studied. It was found that laser blue radiation (3–30 J cm^{-2}) demonstrated a 30 % decrease in electron transport rate in the mitochondrial transport chain, possibly due to photochemical reactions of flavin mononucleotide (FMN) in the respiratory complex II. Red (650 nm) and green (532 nm) radiation effects were negligible. In the presence of nitric oxide, the activity of complexes II–III and IV was decreasing in a concentration-dependent manner, with blue laser irradiation (3 J cm^{-2}) partly restoring cytochrome *c* oxidase activity (complex IV). Experimental facts presented in this paper prove the pivotal role of cytochrome *c* oxidase and its nitrosyl complexes in laser and LED effects.

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