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# Changes in Superoxide Dismutase Activity and Peroxynitrite Content in Rat Peritoneal Macrophages Exposed to He-Ne Laser Radiation

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**Abstract**—The formation of reactive oxygen and nitrogen species by rat peritoneal macrophages induced by a low-intensity He-Ne laser radiation (LR) was studied in this work. It was found that the formation of reactive oxygen species, nitric oxide, and peroxynitrite as well as changes in the activity of superoxide dismutase (SOD) depended to a large extent on the LR dose. In particular, it was found that activation of SOD at low LR doses was accompanied by nitric oxide level increase, while the level of peroxynitrite showed no significant changes. On the other hand, an enhanced LR dose inhibited the enzyme, and this was accompanied by peroxynitrite accumulation. All the measurements were carried out the day after LR treatment. The revealed regularities consequently demonstrate the existence of a deferred LR action on macrophages associated with the production of reactive oxygen and nitrogen species.

*Key words*: laser radiation, phagocytes, active oxygen forms, peroxynitrite

The present paper continues a series of our studies on the cell mechanisms of action of low-power laser radiation (LPLR) [1-4]. Earlier we hypothesized that a free-radical mechanism underlies the stimulating action of LPLR [1, 5, 6]. According to the hypothesis, endogenous porphyrins are primary laser radiation (LR) acceptors that can initiate photoinduced free-radical reactions in cell membranes which are responsible for increased permeability of cell membranes (e.g. those of leukocytes and macrophages) to ions including $Ca^{2+}$. Changes in the intracellular $Ca^{2+}$ concentration can initiate the processes resulting, in particular, in NO-synthase induction and changes in the level of reactive oxygen species (ROS), primarily of superoxide radicals [3, 4, 6]. The modulation of nitric oxide (NO) and superoxide anion radical ($O_2^-$) levels and ratio can be accounted by either a prevailing formation of $ONOO^-$ (producing vasoconstriction) or endothelium relaxing factor (EDRF) production [7].

In our previous papers, we investigated short-term LR effects, i.e., those following immediately after radiation, such as an enhanced formation of radicals by leukocytes and changes in the intracellular calcium concentration. However, the delayed effects (i.e., those observed in a few hours or days after LR) are of no less interest. In the present work, data are presented about the formation of nitric oxide, peroxynitrite, and superoxide anion radical occurring on the next day after the action of LPLR.

## MATERIALS AND METHODS

**Reagents.** The following chemicals were used in this study: Hepes (Flow Laboratories, Scotland); 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR 123) (Molecular Probes, USA); a 4% gentamicin sulfate solution (Joint-Stock Company Belmedpreparaty, Russia); RPMI 1640 medium, 199 medium, and standard Hanks' solution (Institute of Poliomyelitis and Viral Encephalitis, Russia); verografin (Spofa, Czechia); heparin (Richter, Hungary). Superoxide dismutase (SOD), zymosan, NO-synthase inhibitor ($N^G$-monomethyl-L-arginine, L-NMMA), fetal calf serum (FCS), and protoporphyrin IX were purchased from Sigma (USA). 5-Amino-2,3-dihydro-1,4-phthalazinedione (Luminol) and silicon were obtained from Serva (Germany). Naphthylethylenediaminechloride, peptone, NADPH, sulfanilamide, and phenazine methosulfate were from Fluka (Switzerland); trypan blue was a

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product of Chemapol (Czechia); Ficoll was from Pharmacia (Sweden); Fotosens was from NIIOPIK (Russia); ether for narcosis was from Khimmed (Russia). All other chemicals used were domestic products of reagent grade or highest available quality.

**Isolation of rat peritoneal exudate cells.** Ten milliliters of a 2% peptone solution were injected into the peritoneal cavity of 3- to 4-week-old male rats (150-180 g) (Gidrobios Scientific Center, Russia) 3-4 days prior to the experiment [8]. Peritoneal exudate cells were isolated with the method described by Klebanov et al. [2]. Peritoneal cavity cells were collected with a syringe and resuspended in 1 ml of Hanks' solution. The cell suspension contained generally 65 to 75% of macrophages. Cell number was determined with a Gorjaev counting chamber.

**Cultivation of rat peritoneal exudate cells.** Cultivation of peritoneal exudate cells and monocytes was carried out in a $CO_2$ incubator at 37°C in 96-well plates (200,000 cells per well) or in plastic centrifugal tubes ($10^6$ cells/ml). The cells were placed into a solution prepared essentially from medium RPMI 1640 with the addition of a 1% FCS, 10 mM Hepes, and gentamicin sulfate (0.04 mg/ml). The NO-synthase inhibitor L-NMMA, ascorbate, and sodium azide were added immediately into the medium of incubation if necessary. All solutions and incubation media were prepared immediately prior to the experiment.

**Determination of the intracellular production of ROS.** ROS production by peritoneal macrophages was determined by the fluorescence method using the intracellular probe DCFH-DA [9, 10]. A cell suspension or cell lysates obtained by ultrasonic treatment of the suspension were used. In order to saturate cells with fluorophore, they were incubated for 15 min with 5 μM DCFH-DA immediately prior to LR. Preliminary incubation time and fluorophore concentration were chosen according to literature data [11-13]. In the experiments with cell lysates, DCFH-DA was added to the sample immediately prior to radiation.

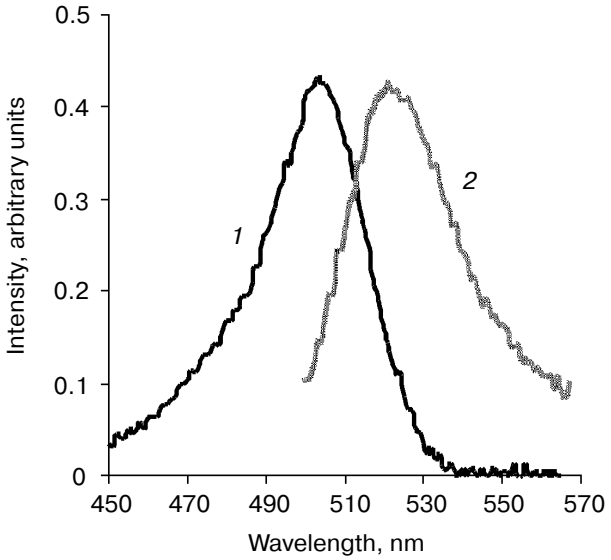


**Fig. 1.** Excitation and fluorescence spectrum of a macrophage suspension of peritoneal exudates of DCFH-DA-supplemented rats. After a 15 min incubation in the presence of 5 μM DCFH-DA at 37°C, macrophages ($10^6$ cells/ml) were exposed to LR, and the spectra of excitation ($\lambda_{em}$ = 521 nm) (curve *1*) and fluorescence ($\lambda_{ex}$ = 501 nm) (curve *2*) of the cell suspension were recorded.

Sample fluorescence was recorded on a MPF-4 spectrofluorimeter (Hitachi, Japan) at the excitation wavelength ($\lambda_{ex}$) of 501 nm and emission wavelength ($\lambda_{em}$) of 521 nm immediately after the exposure to LR. Typical excitation and emission spectra are presented in Fig. 1. The results of fluorescence measurements are presented as a ratio of fluorescence intensity of the irradiated sample to that of non-irradiated sample ($I/I_0$, %).

**Determination of SOD activity.** Immediately after LR, peritoneal exudate cells ($5 \cdot 10^6$) were incubated for 24 h in plastic centrifuge tubes ($10^6$ cells/ml) and then centrifuged for 15 min at 200*g*. The precipitate was resuspended in 1 ml of a phosphate buffer, and the cells were then lysed by adding 1 ml of chloroform to the sample. After intensive stirring, the samples were centrifuged for 15 min at 400*g* for phase separation. Cell lysate-containing aqueous phase was used for further analysis. The assay with nitro blue tetrazolium described by Klebanov et al. [14] was used for determination of SOD activity in the lysate. The reaction of oxidation of β-nicotinamide adenine dinucleotide (NADH) by 5-methylphenazine methosulfate was used as a system for generation of superoxide radicals. Nitro blue tetrazolium was used as an indicator (trap) of superoxide radicals. A commercial enzyme SOD was used as a standard for calculation of the enzyme activity in the experimental samples. The results were expressed in terms of SOD activity units (activity units/$10^6$ cells, AU).

**Determination of the content of nitrite ions ($NO_2^-$) by the Griss method.** Nitric oxide production was determined according to the content of $NO_2^-$ ions in the cell incubation medium by the Griss method described by Shebzukhov et al. [15] and Ding et al. [16]. A sample for determination of $NO_2^-$ concentration contained about 1 ml of distilled water, 150 μl of culture medium, 75 μl of reagent A (1.5% sulfanilamide in 1 N HCl), and 75 μl of reagent B (0.15% naphthylethylenediaminechloride in distilled water). The sample optical density was measured at λ = 548 nm in a SF-46 spectrophotometer (LOMO, Russia). The content of $NO_2^-$ was determined using a calibration plot with different $NaNO_2$ concentrations. Relative $NO_2^-$ content in the cells was expressed as a percentage of $NO_2^-$ content in comparison to cells not exposed to LR.

**Determination of peroxynitrite production.** Peroxynitrite ($ONOO^-$) production was determined by measur-

ing the peroxynitrite-dependent oxidation of dihydrorhodamine 123 (DHR 123) according to Kooy et al. [17]. To determine peroxynitrite production, the cells ($3 \cdot 10^6$), after LR or dark incubation as a control, were incubated in plastic centrifuge tubes ($10^6$ cells/ml) for 24 h. After the incubation, the tubes were centrifuged at 200*g* for 15 min. The precipitate obtained was resuspended in 3 ml of phosphate buffer containing 5 μM DHR 123. After 60 min of incubation at 37°C, the fluorescence of the samples was measured with an MPF-4 spectrofluorimeter. The excitation wavelength ($\lambda_{ex}$) was 500 nm, and the emission wavelength ($\lambda_{em}$) was 536 nm. Peroxynitrite production was estimated using a calibration plot constructed using solutions of known reagent concentrations.

Peroxynitrite was obtained with the technique elaborated and described in [9, 18] by rapid mixing of a 0.6 M $NaNO_2$ solution and 0.6 M $H_2O_2$ (3%) in 0.7 M HCl followed by stabilization in a 0.9 M NaOH solution. Peroxynitrite concentration was determined by a characteristic absorption band at 302 nm ($\varepsilon = 1670\ M^{-1} \cdot cm^{-1}$). Relative peroxynitrite content was expressed as a percentage to that in the cell samples not exposed to LR.

**Irradiation procedure.** A ULF He-Ne laser (Russia) with $\lambda = 632.8$ nm was used as a LR source. A rat peritoneal macrophage suspension ($10^6$ cells) was irradiated in a silicon-coated glass cuvette in a light-proof chamber at 37°C with constant stirring. The radiation beam was directed on the sample via an optical fiber. The mean LR power measured with a RBK 7101 dosimeter (Russia) was 16 mW at the sample surface and the diameter of the beam was 1 cm. In some experiments, an HC8 light filter located immediately before the sample was used to reduce radiation power and, consequently, to control more gradually the exposure time. The average LR power on the sample level was 1.6 mW in the latter case. The control sample was incubated in dark for the same time as the LR duration in the experiment.

## RESULTS AND DISCUSSION

In the first series of experiments, the radiation effect of a He-Ne laser on the intracellular production of ROS was investigated. A fluorescent probe DCFH-DA was used for this purpose [9, 19-22]. The principle of the method is that DCFH-DA, being uncharged and possessing hydrophobic properties, easily penetrates the cell membrane; in the cytoplasm it is hydrolyzed by cytoplasmic esterases to form polar dichlorofluorescein (DCFH) molecules incapable to diffuse through the cell membrane. Under the action of ROS (hydrogen peroxide, peroxynitrite, etc.) DCFH is oxidized and a product is formed possessing fluorescence with excitation wavelength of 501 nm and emission wavelength of 521 nm [10]. According to the literature, it is possible, by using DCFH-DA, to reveal enhanced formation of intracellular ROS under the action of different stimuli. Thus, Bae et al. showed that human epidermoid carcinoma cells A431 stimulation by epidermal growth factor (EGF, 500 ng/ml) was accompanied by enhanced DCFH-DA luminescence [20]. Similar data have been obtained by other authors [21, 22] in experiments on stimulation of pheochromacytoma cells by EGF [22] and rat vessel smooth muscle cells by the platelet growth factor (PDGF) [21]. It was also shown [23] that the probe fluorescence increased if peripheral blood lymphocytes and T-cells were treated with Jurkat phytohemagglutinin and phorbol ether of myristic acid. To detect intracellular ROS, a macrophage suspension was incubated for 15 min in the presence of 5 μM DCFH-DA. The suspension (assay sample) was then irradiated and the probe fluorescence intensity was measured in experimental and control (unexposed to LR) samples.

**Table 1.** Effect of He-Ne laser radiation on the fluorescence of a suspension and lysate of macrophages incubated with dichlorofluorescein diacetate (DCFH-DA)

| Dose, J/cm$^2$ | $I/I_0$, % | |
|---|---|---|
| | suspension | lysate |
| 0 | 100 ± 15 | 100 ± 15 |
| 4.7 | 88.57 ± 13.3 | 103.3 ± 15.5 |
| 14.3 | 102.34 ± 15.3 | 112.2 ± 16.8 |
| 28.6 | 102.9 ± 15.4 | 174 ± 26.1 |

Note: A macrophage suspension was incubated for 15 min in the presence of 5 μM DCFH-DA. The suspension (experimental sample) was then irradiated, and the intensity of probe fluorescence in experimental and control (not exposed to LR) samples was measured. The results were presented as a ratio between the fluorescence intensity of the irradiated sample and that of the non-irradiated control sample ($I/I_0 \times 100\%$).

It was found that LR of the macrophage suspension at doses below 28.6 J/cm$^2$ and in the presence of fluorochrome showed virtually unchanged probe fluorescence intensity (Table 1, second column). Cell lysate was used to enhance the probe fluorescence (Table 1, third column). A dose-dependent enhancement of the probe fluorescence was recorded in this case. The fluorescence increased by 74% at the dose of 28.6 J/cm$^2$, which was maximal in these experiments.

It is known that humans and other animals normally contain free porphyrins in very low concentrations, which, however, increase significantly in the course of development of inflammatory diseases and oncological growth [24]. A low level of endogenous photosensitizers in a peritoneal macrophage suspension is an obvious reason for the fact that DCFH is insensitive to the action of LR on the suspension of the cells. Nevertheless, the situ-

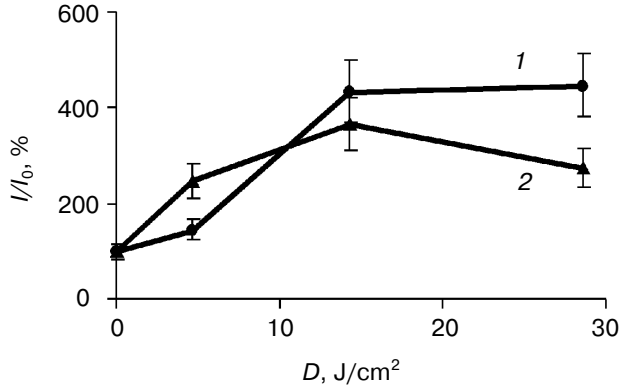


**Fig. 2.** Effect of radiation of a He-Ne laser on the fluorescence of lysate (*1*) and suspension (*2*) of macrophages in the presence of 2′,7′-dichlorofluorescein diacetate and 1 μM Fotosens. Experimental conditions are the same as those indicated in Table 1. Here and below, the data are presented as mean ± standard deviation.

ation changed when macrophages preincubated for 24 h in the presence of the exogenous photosensitizer Fotosens at the concentration of 1 μM were used. It was found that in this case LR brought about a dose-dependent enhancement of DCF fluorescence that reached its maximum at a dose of 14.3 J/cm$^2$ (Fig. 2, curve *2*). The experiment with cell lysate gave similar results (Fig. 2, curve *1*) though the effect was greater: the probe fluorescence increased, at a dose of 28.6 J/cm$^2$, by 347% in the lysate and only by 174% in the cell suspension.

We studied the action of inhibitors of the formation of ROS, sodium azide and ascorbate, on the fluorescence of the lysate of macrophages incubated with DCFH-DA and exposed to LR in the presence of a photosensitizer (Table 2). It was found that sodium azide (30 mM) decreased ROS production in the samples by 1.4-fold and ascorbate (0.5 mM) by 1.8-fold.

These experiments suggest that LR causes formation of ROS inside the cells and this effect is significantly more pronounced in the presence of the photosensitizer Fotosens. It was of interest to disclose which ROS level was changed under the action of LR and what could be the consequences of this change.

In the following series of experiments, we studied the action of LR on macrophage SOD activity and peroxynitrite formation. Simultaneous studies on the action of a He-Ne laser on the activity of SOD and production of nitrite ions and peroxynitrite were carried out on peritoneal exudate cells obtained from one and the same animal. The measurements were performed in cell suspensions incubated for 24 h after LR, and in those unexposed to LR (control samples).

The experimental results showed that radiation in a dose range of 0.36 to 14.1 J/cm$^2$ changed SOD activity in a rat peritoneal exudate macrophage suspension (Fig. 3, curve *3*). The activity of SOD increased at a radiation dose of 0.36 to 0.94 J/cm$^2$ while further increase of the dose to 14.1 J/cm$^2$ resulted in inhibition of the enzyme activity. The maximal SOD activity (170.23 ± 34.04 AU) was observed at the dose of 0.94 J/cm$^2$, where it was 139 times higher than in the cells not exposed to radiation (1.23 ± 0.25 AU) (initial points on the curves correspond to control samples not exposed to LR).

It should be noted that in all experiments the changes in SOD activity in response to LR exhibited the same bell shape as those in nitric oxide production, determined by the Griss method. Indeed, it was shown in the course of the experiment carried out on the same cell population and the results of which are presented in Fig. 3 that both $NO_2^-$ production and SOD activity were elevated in the LR dose range of 0.36 to 0.94 J/cm$^2$ (with maximum of $NO_2^-$ production at 0.36 J/cm$^2$; Fig. 3, curve *1*). Subsequent reduction of SOD activity was accompanied by a decreased concentration of nitrite ions in the macrophage suspension. It should be noted that incubation of the same cells in the presence of cycloheximide (30 μM) abolished the laser-induced enhancement of $NO_2^-$ production and SOD activity (Fig. 3, curves *2* and *4*, respectively). Consequently, it can be assumed that LR of macrophages in this case induced the *de novo* synthesis of proteins (inducible NO-synthase (iNOS) and SOD), which results in the laser-induced enhancement of nitric oxide production and SOD activity (Fig. 3, curves *1* and *3*).

Peroxynitrite production in a macrophage suspension was determined by oxidation of dihydrorhodamine 123 according to the procedure described in [17]. The analysis of nitrite production and SOD activity was carried out in parallel on autologous samples. The experiments showed that peroxynitrite production by the cells changed significantly under LR (Fig. 4, curve *1*). The greatest increase in the production of nitrite was observed in the dose range of 0.1 to 0.94 J/cm$^2$ with the production maximum at a dose of 0.36 J/cm$^2$ (Fig. 4, curve *3*). Further increase in LR dose decreased the level of nitrite. At the same time, a significant increase in peroxynitrite

**Table 2.** Effect of He-Ne laser radiation on the fluorescence of a lysate of macrophages incubated with DCFH-DA in the presence of 1 μM photosensitizer Fotosens and sodium azide or ascorbate

| Sodium azide, mM | $I/I_0$, % | Ascorbate, mM | $I/I_0$, % |
|---|---|---|---|
| 0 | 226.6 ± 23 | 0 | 412.6 ± 41 |
| 30 | 161.7 ± 16 | 0.5 | 232.7 ± 23 |

Note: Radiation dose was 28.6 J/cm$^2$; experimental conditions are the same as those indicated in Table 1.

production can be observed starting with a LR dose equal to or above 0.94 J/cm$^2$ (Fig. 4, curve *1*). Further increase in radiation dose caused an increased peroxynitrite formation up to 333 ± 15% at a dose of 14.1 J/cm$^2$. Obviously, the increase in $ONOO^-$ level occurs simultaneously with a decrease in $NO_2^-$ production (Fig. 4, curve *3*) and lowering of SOD activity (Fig. 3, curve *3*) which probably results in an increased amount of superoxide anion radicals. The latter can react with nitric oxide thus causing peroxynitrite accumulation in the cell suspension.

To support the suggestion that the peroxynitrite formation, as indicated by DHR 123 luminescence, was the consequence of increased NO formation, the cells were incubated in the presence of a 100 μM NO-synthase inhibitor $N^G$-monomethyl-L-arginine (L-NMMA). It was found that the laser-induced $ONOO^-$ formation was reduced in the presence of the inhibitor (Fig. 4, curve *2*). Thus, peroxynitrite production was 333 ± 15% at a radiation dose of 14.1 J/cm$^2$ while its production by the same cells and at the same dose was only 199 ± 12.6% in the presence of L-NMMA. It is important to note that the data in the plot are presented in relative units and the absolute level of peroxynitrite production in the presence of L-NMMA was initially lower than without the inhibitor.

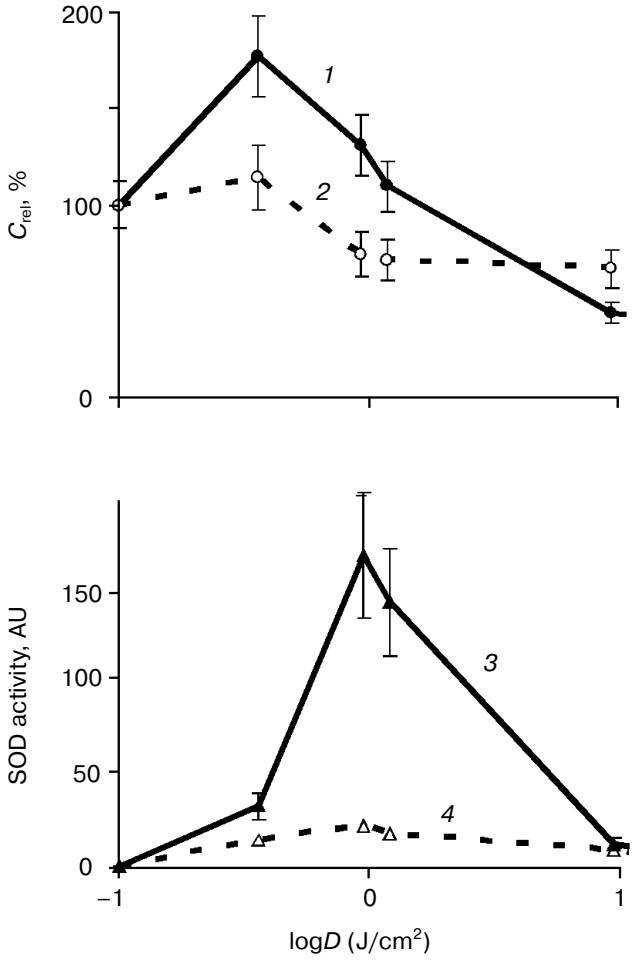


**Fig. 3.** Results of typical experiments and studies on the effect of LR on $NO_2^-$ production and macrophage SOD activity: *1*) $NO_2^-$ production by cells; *2*) the same as (*1*) on incubation in the presence of cycloheximide (30 μM); *3*) SOD activity of cells; *4*) the same as (*3*) on incubation in the presence of cycloheximide (30 μM). The initial points in the curves correspond to control samples (not exposed to radiation). Relative $NO_2^-$ content ($C_{rel}$) in the experimental samples is expressed as a percentage of $NO_2^-$ content in the samples of the cells not exposed to LR. SOD activity is expressed as activity units per million cells (AU).

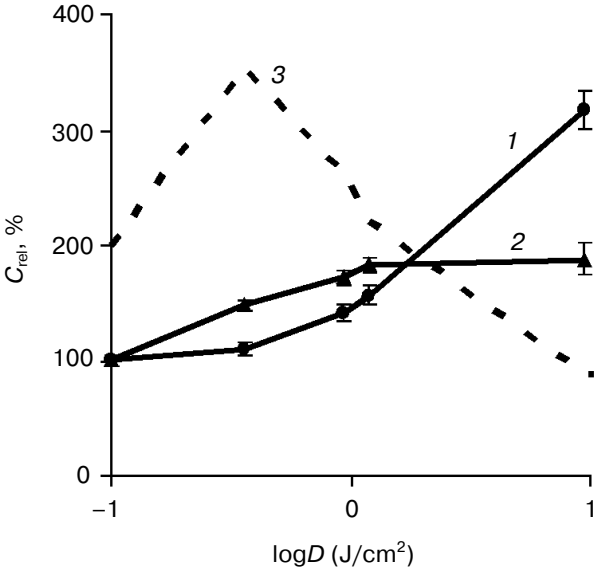


**Fig. 4.** LR dose effect on peroxynitrite and $NO_2^-$ production by macrophages: *1*) peroxynitrite production by intact cells; *2*) peroxynitrite production by the cells incubated in the presence of the NO-synthase inhibitor L-NMMA (100 μM); *3*) formation of $NO_2^-$. The initial points in the curves correspond to control samples (not exposed to radiation). Relative peroxynitrite ($ONOO^-$) and nitrite ($NO_2^-$) content ($C_{rel}$) in the experimental samples is expressed as a percentage of peroxynitrite or $NO_2^-$ content in the samples of the cells not exposed to LR.

Comparing data on SOD activity and peroxynitrite production (Figs. 3 and 4), it can be seen that LR increases both nitric oxide production and SOD activity at the doses below 0.94 J/cm$^2$, while peroxynitrite production increases at doses above 0.94 J/cm$^2$. Apparently, decreased peroxynitrite formation at low LR doses (below 1 J/cm$^2$) is a result of high SOD activity, which removes superoxide and thus suppresses peroxynitrite formation from NO and superoxide radical.

Our experimental observation that the enhancement of peroxynitrite production in laser-irradiated cells occurs against a background of a reduced formation of $NO_2^-$ is worth discussion. Under physiological conditions, peroxynitrite transforms rather quickly to peroxynitrilic acid (ONOOH) which either isomerizes to nitrate ($NO_3^-$)

or dismutates to form nitrogen dioxide ($NO_2$), nitrosodioxyl radical ($ONOO^{\bullet}$), or nitrite and oxygen [25]. The contribution of each of these products is not known and probably depends on the experimental conditions. Nevertheless, some authors are inclined to believe that nitrate ($NO_3^-$) is a principal product of peroxynitrite decomposition. Moreover, peroxynitrite, being a highly reactive compound, can interact with a variety of biologically important compounds in cells. Thus, for example, peroxynitrite can participate in phenylalanine hydroxylation, interaction with $CO_2$ (with a rate constant of $5.88 \cdot 10^{-4}$ $M^{-1} \cdot sec^{-1}$) and thiol groups of proteins, can modify tyrosine residues, etc. [25]. It can therefore be hypothesized that the growth of peroxynitrite formation in the cell changes the relative rate of NO and $NO_2^-$ formation, and the majority of peroxynitrite formed undergoes decomposition to nitrate or interacts with some components of the cell and incubation medium.

In summary, our experiments suggest that certain doses of LR affect not only the synthesis of iNOS by macrophages but also SOD activity and peroxynitrite formation. The *in vivo* SOD activation can (i) be accounted for by antiradical action of LR, since it prevents the reduction by superoxide anion of ferric iron complexes to form non-chelated ferrous ions ($Fe^{2+}$ is known to form radicals in Fenton's reaction and in the reaction of lipid oxidation chain branching). Increased SOD activity may also (ii) promote blood vessel wall relaxation due to a reduced steady-state superoxide concentration and so keeping high the level of endothelium relaxing factor (EDRF), and (iii) increase a bactericidal phagocyte activity due to an enhanced hydroperoxide formation. On the other hand, our experimental data show that beneficial action of low-power LR is observed only within a relatively narrow dose interval, outside of which the effects of LR can be undesirable. For example, reduced SOD production and enhanced peroxynitrite production were observed in our experiments at doses above 1 $J/cm^2$. Peroxynitrite is extremely cytotoxic and may, in particular, suppress NO-induced vasodilatation and provoke vasospasm. At a LR overdose, these phenomena may underlie the effects of the secondary aggravation of pathological states produced by poorly controlled LR frequently observed in clinical practice.

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