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Cellular Effects of Low Power Laser Therapy Can be Mediated by Nitric Oxide

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Background and Objectives: The objective of this study was to investigate the possibility of involvement of nitric oxide (NO) into the irradiation-induced increase of cell attachment. These experiments were performed with a view to exploring the cellular mechanisms of low-power laser therapy.
Study Design/Materials and Methods: A suspension of HeLa cells was irradiated with a monochromatic visible-to-near infrared radiation (600–860 nm, 52 J/m^2) or with a diode laser (820 nm, 8–120 J/m^2) and the number of cells attached to a glass matrix was counted after 30 minute incubation at 37°C. The NO donors sodium nitroprusside (SNP), glyceryl trinitrate (GTN), or sodium nitrite ($NaNO_2$) in the concentration range 5×10^{-9}–5×10^{-4}M were added to the cellular suspension before or after irradiation. The action spectra and the concentration and fluence dependencies obtained were compared and analyzed.
Results: The well-structured action spectrum for the increase of the adhesion of the cells, with maxima at 619, 657, 675, 740, 760, and 820 nm, points to the existence of a photoacceptor responsible for the enhancement of this property (supposedly cytochrome c oxidase, the terminal respiratory chain enzyme), as well as signaling pathways between the cell mitochondria, plasma membrane, and nucleus. Treating the cellular suspension with SNP (5×10^{-5}M) before irradiation significantly modifies the action spectrum for the enhancement of the cell attachment property (band maxima at 642, 685, 700, 742, 842, and 856 nm). The action of SNP, GTN, and $NaNO_2$ added before or after irradiation depends on their concentration and radiation fluence.
Conclusions: The NO donors added to the cellular suspension before irradiation eliminate the radiation-induced increase in the number of cells attached to the glass matrix, supposedly by way of binding NO to cytochrome c oxidase. NO added to the suspension after irradiation can also inhibit the light-induced signal downstream. Both effects of NO depend on the concentration of the NO donors added. These results indicate that NO can control the irradiation-activated reactions that increase the attachment of cells. Lasers Surg. Med. 36:307–314, 2005.

Key words: adhesion; action spectrum; cytochrome c oxidase; nitric oxide; low power laser therapy

INTRODUCTION

The free radical nitric oxide (NO) is an important inter- and intracellular messenger involved in a variety of physiological and pathophysiological conditions [1]. Experimental studies performed so far to investigate the possible role of NO in the effects of low power laser therapy bear witness to the involvement of NO at least in two cases.

The analgesic effects of red and near-infrared laser radiation were well documented in various laboratory and clinical experiments (see review [2]). NO was found to mediate the fast and transient analgesic effects caused by low-power laser radiation at 904 nm [3].

NO is involved in the radiation-induced mesenteric arteriolar vasodilatation and the subsequent increase in the microcirculatory blood flow [4]. Irradiation at different wavelengths induces vasodilatation in blood-perfused vessels but not in saline-perfused ones [5]. It was suggested that NO–hemoglobin might serve as a light-sensitive store of NO in red blood cells from which it was released by irradiation [6].

NO also accomplishes regulatory tasks in cultivated cells. Addition of NO donors to cells cultivated in vitro increases or inhibits their proliferation and cell-to-matrix adhesion [7–13]. Energy production, stimulation of the biogenesis of mitochondria, and apoptosis can be controlled by NO [14,15].

Investigations into the possible regulatory role of NO in irradiated cells have gained no attention so far. A pilot experiment has indicated that high concentrations of NO donors inhibit the attachment of irradiated cells [16]. The existence of an irradiation-controlled mitochondrial NO signaling pathway in cells has recently been explored [17].

The surface of a cell and its adhesive interactions are involved in the regulation of such processes as embryogenesis, cell growth and differentiation, wound repair, formation of metastases, to name but a few [18]. The

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present study is aimed at studying, on a cell-to-glass adhesion model, the irradiation-controlled cellular signaling pathways including cytochrome c oxidase and NO. We use three NO donors (sodium nitroprusside, SNP; sodium nitrite, $NaNO_2$; and glyceryl trinitrate, GTN) in a wide concentration range (10^{-9}–10^{-4}M) in combination with visible-to-near infrared (NIR) radiation of various wavelengths (600–860 nm) and fluences (8–120 J/m^2). The experimental approach in this study is the recording of the light action spectra of the cells, rather than their absorption spectra. This approach offers an important benefit. The recording of the absorption spectra of living cells in the far red-to-NIR region under physiological conditions, as well as the interpretation of these spectra, is a problem because of the technical difficulties associated with the broad overlapping of the weak absorption bands of the redox carriers in this region of the spectrum and the extremely fast electron transfer in the course of the redox cycles. Light action spectra are much easier to record, and such spectra can provide information about the primary photoacceptor. Recall that any graph representing a biological response as a function of radiation wavelength is called an action spectrum, and the action spectrum coincides (to certain limits) with the absorption spectrum of the photoacceptor molecule involved [19]. We intend to demonstrate that NO can control the irradiation-activated reactions that increase the attachment of cells to a glass matrix.

MATERIALS AND METHODS

Cells

HeLa cells, obtained from the Institute of Virology, Moscow, Russia, were cultivated as monolayer in closed Carrell vials (diameter 45 mm), at 37°C in 5 ml of RMPI-1640, with 10% of fetal bovine serum, and 100 U/ml of penicillin and streptomycin. 1.5×10^6 cells were plated per vial and grown for 72 hours (middle-log phase). The HeLa culture used is characterized by anchorage-dependent growth and forms confluent monolayer. All chemicals used for cell cultivation were purchased from ICN Pharmaceutical, Amsterdam, The Netherlands.

Cells were harvested using 0.02% Versene solution (37°), and suspension for irradiation was prepared in medium RMPI-1640 containing 10% of bovine embryo serum. Cell culture processing was performed in dark or under dim natural light. Extraneous illumination (sunshine or artificial light) was avoided.

Light Sources

Monochromatic radiation was obtained by means of a monochromator designed by Dr. A. Lifshits at the Institute of Spectroscopy, Russian Academy of Sciences, Troitsk, to have bandwidths of 400–700 and 540–1,050 nm. The monochromator was operated in accordance with an autocollimation scheme. A single-slit 1.2 mm wide was used to direct radiation both into and out of the monochromator. Dispersion amounted to 8 nm/mm. Thus, the exit radiation bandwidths came to 10 nm (full wide at half maximum, FWHM). The dispersive element used was a diffraction grating of 1,200 lines per millimeter. To obtain radiation in region 600–860 nm, the second order of the grating, a thermal filter and a 600 nm cut-off glass filter, placed between the radiation source (a stabilized 250 W high-pressure xenon arc lamp) and the monochromator, were used. The source radiation was focused with a positive lens and delivered by a fiber light guide to the upper part of the slit. After diffraction of the beam, the monochromatic radiation was exited from the lower part of the slit and transmitted by another light guide. In the range of wavelengths 600–860 nm, the output power of the monochromator and the power measured after the sample were constant. Power meter Fieldmaster (Coherent, Portland, OR) suitable for measuring powers in microwatt range was used for the measurements. Light intensity in experiments was 1.3 W/m^2, dose 52 J/m^2, irradiation time 40 seconds. The light spot fully covered the vial with cell suspension (0.38 cm^2).

Radiation at 820 nm was obtained from a GaAlAs light diode (Polyus, Moscow, Russia). The technical characteristics of this light source were as follows: $\lambda = 820 \pm 10$ nm, pulse repetition frequency 10 Hz, pulse width 20 milliseconds, and duty factor (pulse duration-to-pulse period ratio), 20%. Irradiation was performed through an optical fiber, so that a homogenous light spot covered the exposed suspension surface (0.38 cm^2). The radiation intensity at the suspension level was 3.54 W/m^2, and the radiation dose varied from 8 to 120 J/m^2 (exposure time, from 10 to 170 seconds). Intensity measurements were taken using power meter M404 (Spectra-physics, Mountain View, CA).

Irradiation

The samples of cellular suspension were irradiated in glass sample cells. These cuvettes were made as follows. Two glass rings with ground in edges (inner diameter 0.7 cm, height 0.4 cm, volume 130 μl) were stuck on a microscope slide with 5-cm distance between them. Both wells were filled with cell suspension (85,000 cells per vial). Always, one of the vials was irradiated and another one was used as control. Optimal irradiation conditions (shape and dimensions of the vial, number of cells per vial) were developed in a special series of experiment [20]. Irradiation was performed at room temperature in the dark. The control well was protected from light during irradiation experiment. The new pair of sample cells was used for every measurement.

Measurement of Cell–Glass Adhesion

The criterion by which changes in the adhesion properties of the cell membrane were judged was the number of cells that attached themselves to the bottom of the cuvette in the course of 30 minutes at 37°. This time was chosen according to experiments describing time-course of attachment of HeLa cells to the glass substratum in our experimental condition [20]. In control culture, $42.5\% \pm 2.5\%$ of cells is adhered to the surface after 30 minute incubation. Thus, in these conditions the

stimulatory and inhibitory effects of the irradiation and chemicals could be measured under the same conditions. After incubation, the nutrient medium was removed, and the cuvettes were washed with warm (37°) Hanks' solution to remove non-attached cells. The attached cells were trypsinized, and their number was counted with a haemocytometer. Each data point represents the mean of at least 10 independent measurements.

NO Donors

SNP and $NaNO_2$ were purchased from Sigma Chemical Co (St. Louis, MO). GTN (commercial name Nitrocine) was from Schwarz Pharma AG (Monheim, Germany). Ten microliters of freshly prepared solution of a chemical in non-colored Hanks' solution (37°) was added to each cuvette immediately before or after irradiation; 10 μl of Hanks' solution without the chemical was added to the control cuvettes. Stock solutions of the chemicals were made up freshly before the experiments and protected from light. The toxicity of the chemicals was assessed using the Trypan blue exclusion test: all concentrations used were found to be non-toxic under our experimental conditions (viability of cells $>95\%$). The solutions of inhibitors did not show any absorption bands in the optical region under study (checked by recording the absorption spectra).

Statistical Analysis

The results obtained were statistically processed by means of the Graphpad Prism 4.0 (San Diego, CA) program package and expressed in terms of the mean value ± SEM from 10 measurements. $P < 0.05$ was considered significant using the ANOVA and Student's *t*-tests. Deconvolution of action spectra with Lorentzian fitting and baseline corrections were made using the Origin 7.5 (Northampton, MA) software program.

RESULTS

Addition of NO Donors to Cell Suspension in Certain Concentration Range Does not Affect Cell Attachment to the Glass Matrix

In our experimental conditions 42.5% ± 2.5% of the total number of cells in a cuvette (85,000) attached to the glass bottom of the vial. Figure 1 presents the dependencies of percent of adhered cells on the concentration of added chemicals SNP, GTN, and $NaNO_2$ without irradiation. In our experimental conditions, SNP and $NaNO_2$ in concentration range from 5×10^{-9} to 5×10^{-4}M do not influence cell attachment. GTN do not influence cell attachment in concentration range from 4×10^{-9} to 4×10^{-7}M. The percentage of attached cells increases at higher GTN concentrations (from 4×10^{-6} to 4×10^{-4}M) in concentration-dependent manner.

In our following experiments we use NO donors in concentrations which were found in these experiments not to influence cell adherence. Decomposition of NO donors under irradiation is not expected due to absence of absorption bands in red-to NIR optical region.

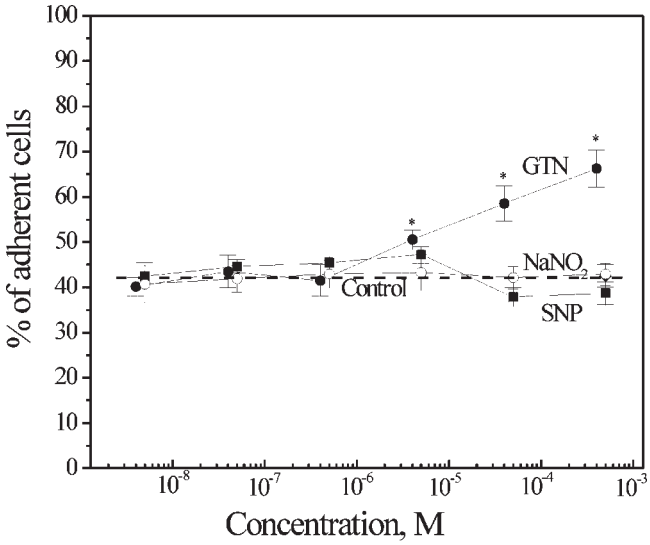

Fig. 1. Dependencies of cell attachment on concentration of nitric oxide (NO) donors added to cell suspension in the absence of irradiation: sodium nitroprusside (SNP), glyceryl trinitrate (GTN), and sodium nitrite ($NaNO_2$). The dashed line shows the attachment of control cells. Asterisks indicate difference from control ($P < 0.05$).

Red-to-NIR Radiation Increases Cell Attachment in Wavelength-Dependent Manner and NO Modifies This Action Spectrum

The percentage of cells attached to the glass surface is increased upon irradiating the cell suspension samples with certain wavelengths. Figure 2A represents the measured light action spectrum as well as the deconvolution of this spectrum with Lorentzian fitting. The light action spectrum of cell adherence to glass (Fig. 2A) is characterized by a single peak at 619 nm, triplet peaks at 657, 675 (main peak), and 699 nm (a weak shoulder), doublet peaks at 740 and 760 nm (main peak) as well as by quartet peaks at 800, 820, 840, and 860 nm (Table 1). In this quartet, the main peak is at 820 nm and peaks at 800, 840, and 860 nm are resolved as shoulders.

Figure 2B presents the measured action spectrum as well as deconvolution of this spectrum with Lorentzian fitting when SNP (5×10^{-5}M) is added to cell suspension samples before the irradiation. This new action spectrum is characterized by a single peak at 642 nm, doublet peaks at 685 and 700 nm (main peak), single peak at 742 nm (with shoulder at 770 nm), and doublet peaks at 842 (main peak) and 856 nm.

Table 1 presents the results of comparison of two action spectra and underlines the changes in peak positions occurring due to SNP addition. The results of this experiment evidence that NO added before the irradiation causes substantial changes in absorption of the primary photoacceptor.

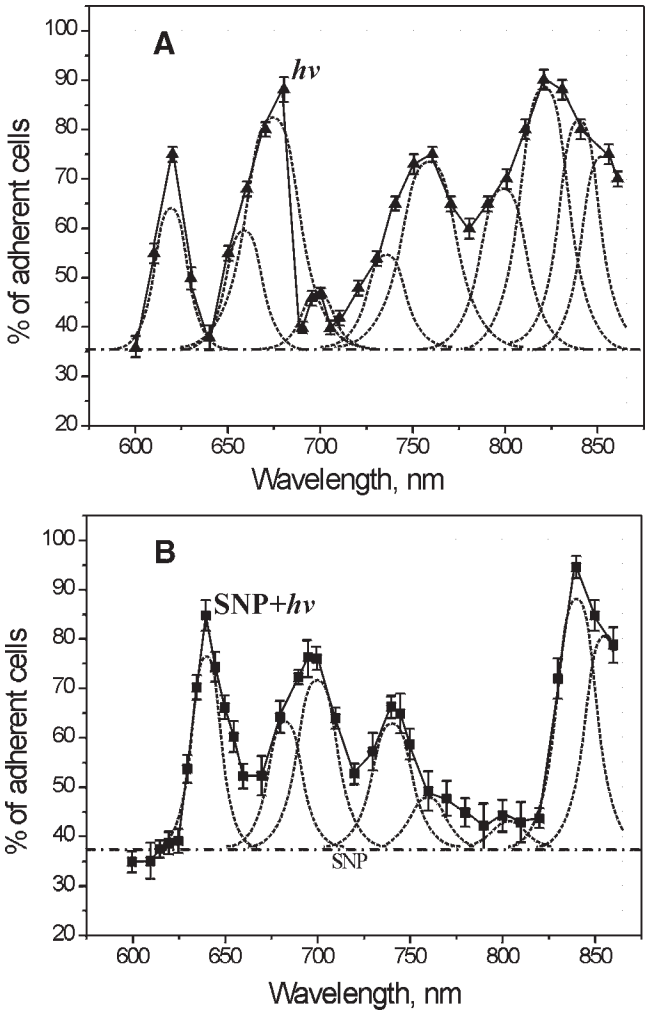

Fig. 2. Dependence of cell attachment on wavelength used for irradiation of cell suspensions (CW light, fluence 52 J/m^2, intensity 1.3 W/m^2, irradiation time 40 seconds, adhesion measurements performed 30 minutes after irradiation) (**A**) without, and (**B**) with SNP (5×10^{-5}M) added before irradiation.

Modification of Cell Attachment by NO Depends Both on Fluence and Concentration of NO Donors

In the action spectroscopy experiment (Fig. 2), we used SNP in concentration of 5×10^{-5}M and the wavelength of radiation was changed. In the next series of experiments we examined the situation when SNP as well as GTN and $NaNO_2$ were taken in various concentrations from 4×10^{-9} to 5×10^{-4}M but only one wavelength (820 nm, i.e., the wavelength of one maximum in the action spectrum, Fig. 2A) and one fluence (60 J/m^2) were used. The fluence 60 J/m^2 was chosen because this provides the maximal effect for the increase of cell attachment at the wavelength of 820 nm (curve labeled *hv* in Fig. 3). Note that pulsed radiation provides less stimulative effect ($64.5\% \pm 2.0\%$) when used in the optimal fluence (60 J/m^2, maximum of the curve labeled *hv* in Fig. 3) as compared with the continuous wave (CW) light used in action spectroscopy (at 820 nm and fluence 52 J/m^2 percent of adhered cells is $90.0 \pm 2.0\%$, Fig. 2A). This finding corroborates the data about different magnitude of a biological response of cells to CW and pulsed light of the same fluence and wavelength [21]. Pulsation of the light has no principal importance in the present experiments insofar as the wavelength is the same.

The increase in percentage of adhered cells as the dependence on the light fluence is labeled *hv* in Figure 3. SNP in concentration 5×10^{-7}M does not affect cell attachment at any fluence (curve labeled 5×10^{-7} SNP+*hv* in Fig. 3) as compared to curve labeled *hv*. SNP in concentration 5×10^{-5} M eliminates the light-induced cell attachment stimulation fully at fluence 60 J/m^2 only. At lower and at higher fluences, a gradual elimination of radiation-induced stimulation occurs. At the fluences of 16 and 120 J/m^2 (curve labeled 5×10^{-5} SNP+*hv* in Fig. 3), the percent of attached cells is comparable to that in samples irradiated at optimal fluence of 60 J/m^2. SNP at a high concentration of 5×10^{-4}M inhibits cell attachment, maximal inhibitive effect occurs at 60 J/m^2 (curve labeled 5×10^{-4} SNP in Fig. 3).

Figure 4 presents the dependencies of percentage of adhered cells on concentration of SNP, $NaNO_2$, or GTN added before (Fig. 4A) or after (Fig. 4B) irradiation at 820 nm and 60 J/m^2. In the control suspension, $42.5\% \pm 2.5\%$ of cells is attached. Irradiation at 820 nm (60 J/m^2) increases the percent of attached cells to $64.5\% \pm 2.0\%$. It means that a new subpopulation (22%) is attached due to the irradiation.

Treatment of cell suspension with NO donors in various concentrations before the irradiation influences the percentage of cells attached (Fig. 4A). It is possible to distinguish three regions of concentrations with different types of action. First, at low concentrations of NO donors (from 4×10^{-9} to approximately 5×10^{-8}M), percents of cells attached are comparable to this in the control. It means that NO do not allow a subpopulation ($\sim$20%) of cells to adhere. In other words, in presence of low concentrations of NO the effect of radiation (stimulation of cell attachment) does not appear. Secondly, NO donors in medium concentration range (approximately between 10^{-7} and 10^{-5}M) do not influence cell attachment. In this case, the percentage of attached cells is close to the percent of cells attached under irradiation without chemicals added. Thirdly, at higher concentrations (approximately higher than 5×10^{-5}M), NO donors gradually decrease percentage of cells stimulated by light to attach. SNP causes even an inhibition of cell attachment below the control level. Three curves in Figure 4A do not coincide exactly and the concentration regions are defined rather roughly. This is probably caused by differences in NO release mechanisms and thereby NO fluxes provided by different donors [22–26].

The situation is opposite to that described above when the cell suspensions are first irradiated at 820 nm (60 J/m^2) and then NO donors are added at various concentrations (Fig. 4B). NO donors added after the irradiation do not obstruct cell attachment stimulation both at low concentrations near 10^{-9}–10^{-8}M and at high concentrations near

TABLE 1. Comparison of Peak Positions in Two Action Spectra (Control and SNP Added)

Control spectrum		SNP (5×10^{-5}M) added before the irradiation		Changes in peak position due to SNP addition
Characteristics	Peak positions, nm	Characteristics	Peak positions, nm	
Single	**619**		—	Disappeared
		Single	**642**	New peak with the same intensity as the peak at 619 nm in control spectrum
	657		—	Disappeared
Triplet	**675**	Doublet	685	Shift + 10 nm, decrease in intensity two times
	699 w. sh		**700**	New and intensive band instead of weak shoulder of the peak at 675 nm in control spectrum
Doublet	740	Doublet	**742**	No changes in the intensity, but this is a new distinct band
	760		~770 w. sh	From this peak only a shoulder keeps
	800 s. sh.		—	Disappeared
Quartet	**820**	Doublet	—	Disappeared
	840 s. sh.		**842**	No change
	860 s. sh.		856 s. sh.	No change

s. sh., strong shoulder; w. sh., weak shoulder.
Peak positions are calculated from Lorentzian fitting data; changes < 5 nm are considered to be nonsignificant. Main peaks are marked bold.

10^{-5}–10^{-4}M. There is an concentration range between approximately 5×10^{-8} and 5×10^{-6}M: added in these concentrations donors eliminate light-induced cell attachment stimulation. Percent of attached cells is comparable to that in the control when the concentration of NO donors is near 5×10^{-7}M. It means that NO deliberated at this point fully obstructs a subpopulation of cells (~20%) to adhere after cells have percept the light signal. Again as in the previous case (Fig. 4A), the concentration ranges here are not exactly the same for all three donors (Fig. 4B).

Complicated dependencies of cell attachment on irradiation fluence at different concentrations of NO donors (Fig. 3) as well as those on concentration of NO donors when irradiating at a constant dose (Fig. 4A,B) indicate that both factors (irradiation and NO) have a subtle influence upon a reaction channel (cellular signaling cascade) between the photoacceptor and the response of cells to irradiation (cell attachment).

DISCUSSION

The number of HeLa cells attached to glass substrate increases after irradiation of cell suspensions with monochromatic visible-to-NIR radiation, which means that a new subpopulation of cells enter an adhesive state due to irradiation. The percentage of the subpopulation made capable of adhering to glass depends on the parameters of the radiation used, first of all on wavelength (existence of the action spectrum, Fig. 2A) and on fluence (Fig. 3, the curve labeled *hv*).

Maximal amount of cells in this subpopulation in our experimental conditions under radiation at 820 nm is ~22% (Figs. 3 and 4). An analysis of the results presented in Figures 3 and 4 indicates that NO in combination with irradiation also influences attachment of a cell population in amount approximately 20%. There is no reason to exclude the possibility that NO affects the same

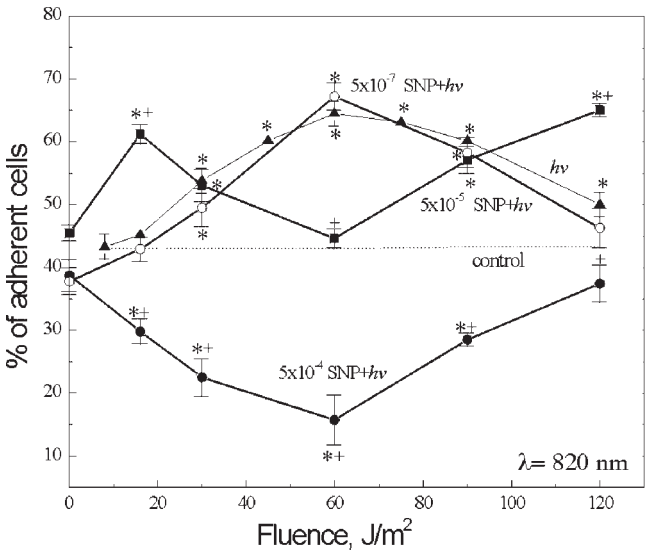

Fig. 3. Effects of SNP on cell attachment in irradiated cells. SNP in concentrations 5×1^{-4}, 5×10^{-5}, and 5×10^{-7}M was added to the cell suspensions before irradiation at various fluences (pulsed radiation at $\lambda = 820$ nm, intensity 3.54 W/m^2, irradiation time 10–170 seconds). The dashed line shows the attachment of control cells; the curves labeled *hv* indicate the dose-dependence of light action. Asterisks indicate difference ($P < 0.05$) from control (*) or from curve labeled *hv* (+).

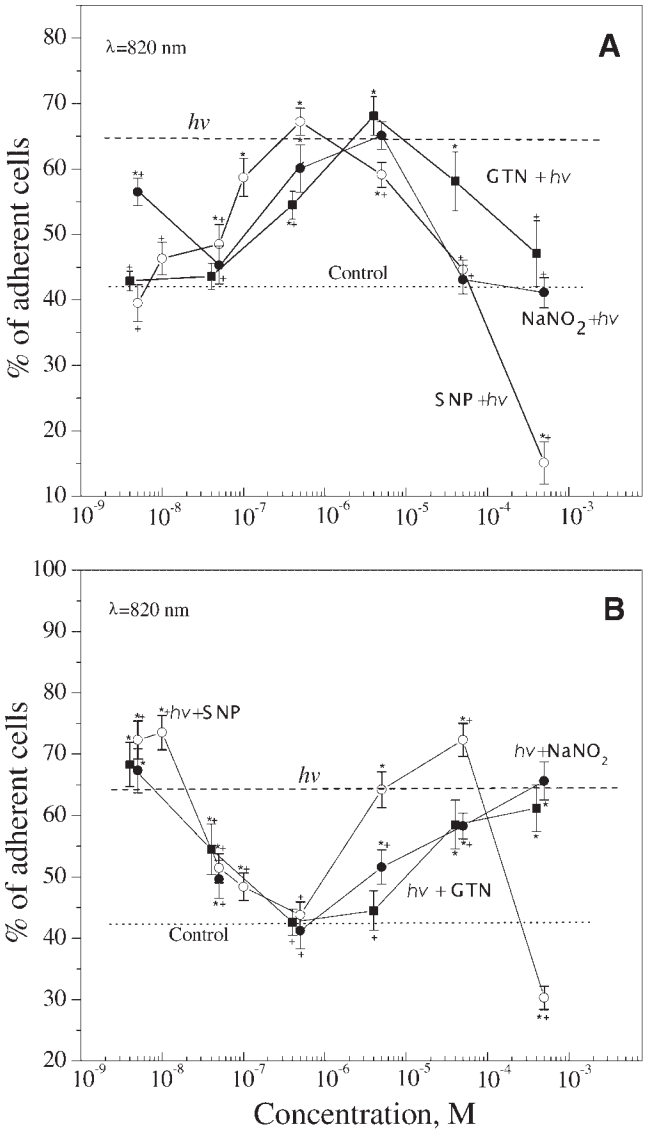

Fig. 4. Dependence of cell attachment on concentration of SNP, GTN, and $NaNO_2$ added to the cell suspension (**A**) before or (**B**) after irradiation at $\lambda = 820$ nm, 60 J/m^2. The dashed line labeled *hv* shows the attachment of cells irradiated at $\lambda = 820$ nm, 60 J/m^2 without chemicals added. The dotted line indicates the attachment of control cells. Asterisks indicate difference ($P < 0.05$) from control (*) or from curve labeled *hv* (+).

subpopulation that was stimulated to adhere by irradiation. Recall that NO donors without irradiation have no influence on attachment of cells in concentrations (Fig. 1) used in experiments, results of which are presented in Figures 3 and 4. So, both agents, photons and NO, quite probably affect the same subpopulation of cells and the final result (change in percent of attached cells) depends both on radiation fluence (Fig. 3) as well as on concentration of NO donors and application order of irradiation and NO donors (Fig. 4).

Our data evidences that the irradiation activates a reaction channel from the photoacceptor (putatively cytochrome c oxidase [17,27]) that leads to the regulation of cell attachment. An analysis of data in Figure 4A allows to suggest that NO donors applicated before the irradiation in low ($\sim 10^{-9}$–10^{-8}M) as well as in high ($\sim 10^{-5}$–10^{-4}M) concentrations block this light-induced reaction channel. NO block does not occur in medium concentration range of the donors ($\sim 10^{-7}$–10^{-8}M). The block created by NO appears on the level of the primary photoacceptor as evidenced by the results of action spectroscopy (Fig. 2).

The situation is inverse when NO donors are added after the irradiation. NO donors in low and high concentrations do not influence attachment of irradiated cells. NO donors only in medium concentration range ($\sim 10^{-7}$–10^{-6}M) cause a decrease of percentage of cells stimulated to attach by irradiation (Fig. 4B). It means that NO released from the donors applied in these concentrations can inhibit a light-activated reaction channel also downstream. It appears that different action mechanisms to influence cell attachment by NO are in work in dependence of concentration of NO donors and application order.

In addition to multiple regulatory functions on organism level [1], NO has been recognized as a potential signaling molecule controlling cell respiration [14,15,28–32]. It is known that NO in nanomolecular and low micromolecular concentration range reversibly inhibits cytochrome c oxidase in a competitive manner with oxygen [15,28,32]. However, concentration of NO as well as NO flux, which achieves the respiratory chain are not known in situ when NO donors are added to cell suspension. Also, it is known that not total NO released but NO flux is important in reactions inside the cells [22]. Three NO donors used in our study differ from each other by NO release mechanisms and rates [23–26]. Concentrations of NO donors added to cells are apparently higher than NO concentrations within the cells and mitochondria, but the exact values of it are not known in our experiments. It is difficult to discuss why different concentrations of NO donors provide for different effects when applied together with the irradiation (Figs. 3 and 4). One reason is that at high concentrations, NO inhibits many components of the respiratory chain, the oxygen binding site of cytochrome c oxidase included [14]. Also, NO can be substrate, inhibitor or effector of cytochrome c oxidase, depending on cellular conditions [28].

The light action spectrum of cell attachment enhancement shown in Figure 2A is believed to mirror the absorption of one (as yet unidentified) redox intermediate of cytochrome c oxidase [17,27]. Changes in absorption of the photoacceptor molecule (reflected by the action spectra in our case) are commonly caused by structural and conformational modifications of this molecule. The present type of experiment can not evaluate the type of interaction occurring between NO and the photoacceptor molecule. Two reactions of NO fast enough to be relevant to the cytochrome c oxidase turnover are those between NO and reduced haem a_3 and between NO and oxidized Cu_B. Both these reactions can play a role in the inhibition of enzyme activity by NO [28], but important from our point of view is

the first one as far as only the NO–haem complex is photolabile [33,34]. It is known that the light sensitive, reversible inhibition by NO occurs at high electron flux only (reduced enzyme) [28]. Recall that monochromatic radiation in visible region can cause photoreduction of cytochrome c oxidase both in soluble preparations as well as in isolated mitochondria [35–41]. Purified cytochrome c oxidase both in soluble preparations as well as in isolated mitochondria were photoreduced by green light [40] similarly by UV radiation ($\lambda < 300$ nm) [41]. Pulsed laser light at 532 nm caused redox absorbance changes and electrogenic events in partly reduced cytochrome c oxidase, indicating increased electron transfer from Cu_B to oxygen [41,42].

An analysis of the action spectra (Table 1) indicates that NO causes a new internal distribution of electrons in redox centers of the photoacceptor molecule. A comparison of spectra in Figure 2A,B shows that all system of bands characteristic to one redox state of the photoacceptor smoothly shifts in the presence of NO to an alternate redox state of the photoacceptor. An important feature of this rearrangement is closing of some reaction channels between redox centers and opening of some new ones with similar characteristics of band intensity, shape of contour, and bandwidth.

The signaling pathways (reaction channels) that are involved in signal transduction between the photoacceptor in the respiratory chain, the nucleus, and the plasma membrane are not established yet. Suggestions have been made to explain these complicated signaling pathways via regulation of cellular homeostasis parameters like pH_i and cellular redox potential, increase in ATP and Ca^{2+} concentration, expression of redox-sensitive factors like NF-κβ [27]. A recent research result using cDNA microarrays indicates that irradiation with red light regulates expression of genes in human fibroblasts. Several genes related to antioxidation and mitochondria energy metabolism are also expressed upon irradiation [43].

It is found in this study that NO donors applied to cell suspension before irradiation eliminate increase of attachment of cells to the glass matrix supposedly via binding of NO to the cytochrome c oxidase. NO added to the cells after irradiation can inhibit light-induced signal also downstream. Both effects of NO depend on concentration of NO donors added as well as light fluence.

REFERENCES

1. Moncada S, Palmer RMJ, Higgs EE. Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol Rev 1991;43: 109–142.
2. Tuner J, Hode L. Laser therapy. Grängesberg, Sweden: Prima Books Sweden; 2001.
3. Mrowiec J, Sieron A, Plech A, Cieslar G, Biniszkiewicz T, Brus R. Analgesic effect of low-power infrared laser radiation in rats. In: Bottiroli G, Karu TI, Lubart R, editors. Effects of low-power light on biological systems III. Bellingham: SPIE Proc.; 1997;3198:83–89.
4. Maegawa Y, Itoh T, Hosokawa T, Yaegashi K, Nishi M. Effects of near-infrared low-level laser irradiation on microcirculation. Lasers Surg Med 2000;27:427–437.
5. Schwengel RH, Gregory KW, Hearne SE, Scott HJ, Beauman GJ, Mergner WJ, Caplin JL, Ziskind AA. Characterization of pulsed-dye laser-mediated vasodilatation in a rabbit femoral artery model of vasoconstriction. Lasers Surg Med 1993;13: 284–295.
6. Vladimirov Y, Borisenko G, Boriskina N, Kazarinov K, Osipov A. NO-hemoglobin may be light-sensitive source of nitric oxide both in solution and in red blood cells. J Photochem Photobiol B:Biol 2000;59:115–122.
7. Garg UC, Slassid A. Nitric oxide-generating vasodilators inhibit mitogenesis and proliferation of BALB/C3T3 fibroblasts by a cyclic GMP-independent mechanism. Biochem Biophys Res Commun 1990;171:474–479.
8. Garg UC, Devi L, Turudorf H, Goldfrank LR, Bansinath M. Effect of nitric oxide on mitogenesis and proliferation of cerebellar glial cells. Brain Res 1992;592:208–212.
9. Motohashi S, Kasai K, Banba N, Hattori Y, Shimoda S. Nitric oxide inhibits cell growth in cultured human thyrocytes. Life Sci 1996;59:PL227–PL234.
10. Du M, Islam HM, Lin L, Ohmura Y, Moriyama J, Fujimura S. Promotion of proliferation of murine BALB/c3T3 fibroblasts mediated by nitric oxide at lower concentrations. Biochem Mol Biol Int 1997;41:625–631.
11. Gansauge S, Gansauge F, Nussler AK, Rau B, Poch B, Schoeneberg H, berger H. Exogeneous, but not endogeneous, nitric oxide increases proliferation rates in senescent human fibroblasts. FEBS Lett 1997;410:160–164.
12. Ulibarri JA, Mozdziak PE, Schultz E, Cook C, Best TM. Nitric oxide donors, sodium nitroprusside and *S*-nitro-*N*-acetylpenicillamine, stimulate myoblast proliferation in vitro. In Vitro Cell Dev Biol Anim 1999;35:215–218.
13. Wangsiripaisan A, Gengaro PE, Nemenoff RA, Ling H, Edelstein CL, Schrier W. Effect of nitric oxide donors on renal tubular epithelial cell–matrix adhesion. Kidney Int 1999;55:2281–2288.
14. Brown GC. NO says yes to mitochondria. Science 2003;299: 838–839.
15. Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochem Biophys Acta 2001;1504:46–57.
16. Karu TI, Pyatibrat LV, Kalendo GS. Donors of NO and pulsed radiation at $\lambda = 820$ nm exert effects on cells attachment to extracellular matrices. Toxicol Lett 2001;121: 57–61.
17. Karu TI, Pyatibrat LV, Afanasyeva NI. A novel mitochondrial signaling pathway activated by visible-to-near infrared radiation. Photochem Photobiol 2004;80:366–372.
18. Horton MA, editor. Molecular biology of cell adhesion molecules. Glasgow: John Wiley; 1996.
19. Hartmann KM. Action spectroscopy. In: Hoppe W, Lohmann W, Marke H, Zielger H, editors. Biophysics. Heidelberg: Springer-Verlag; 1983. pp 115–151.
20. Karu TI, Pyatibrat LV, Kalendo GS, Esenaliev RO. Effects of monochromatic low-intensity light and laser irradiation on adhesion of HeLa cells in vitro. Lasers Surg Med 1996; 18:171–177.
21. Karu TI, Ryabykh TP, Letokhov VS. Different sensitivity of cells from tumor-bearing organisms to continuous-wave and pulsed laser radiation ($\lambda = 632.8$ nm) evaluated by chemiluminescence test. III. Effect of dark period between pulses. Lasers Life Sci 1997;7:141–156.
22. Wink DA, Cook JA, Krishna MC, Hanbauer I, DeGraff W, Gamson J, Mitchell IB. Nitric oxide protects against alkyl peroxide-mediated cytotoxicity: Further insights into the role nitric oxide plays in oxidative stress. Arch Biochem Biophys 1995;319:405–407.
23. Feelish M. The use of nitric oxide donors in pharmacological studies. Naunyn-Schmiedeberg's Arch Pharmacol 1998;358: 113–122.
24. Yamamato T, Bing R. Nitric oxide donors. PSE BM 2000;225: 200–206.
25. Adami A, Crivellente F, Carcereri De Prati A, Cavalieri E, Cuzzolin L, Tommasi M, Suzuki H, Benoni G. Biotransformation and cytotoxic properties of NO-donors on MCF7 and U251 cell lines. Life Sci 1998;63:2097–2105.
26. Ignarro LJ, Napoli C, Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide. Circ Res 2002;90:21–28.

27. Karu T. Primary and secondary mechanisms of action of visible-to-near IR radiation on cells. J Photochem Photobiol B: Biol 1999;49:1–17.
28. Cooper CE. Nitric oxide and cytochrome oxidase: Substrate, inhibitor or effector? Trends Biochem Sci 2002;27:33–39.
29. Sarti P, Giuffrè A, Barone MC, Forte E, Mastronicola D, Brunori M. Nitric oxide and cytochrome c oxidase: Reaction mechanisms from the enzyme to the cell. Free Radic Biol Med 2003;34:509–520.
30. Mastronicola D, Genova HL, Arese M, Barone MC, Giuffre A, Bianchi C, Brunori M, Lenaz G, Sarti P. Control of respiration by nitric oxide in Keilin–Hartree particles, mitochondria and SH-SY5Yneuroblastoma cells. Cell Mol Life Sci 2003;60: 1752–1759.
31. Torres J, Cooper CE, Wilson MT. A common mechanism for the interaction of nitric oxide with the oxidized binuclear centre and oxygen intermediates of cytochrome c oxidase. J Biol Chem 1998;273:8756–8766.
32. Brown GC, Cooper CE. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal cytochrome c oxidase respiration by competiting with oxygen at cytochrome c oxidase. FEBS Lett 1994;356:295–298.
33. Boelens R, Rademaker H, Pel R, Wever R. EPR studies of the photodissociation reactions of cytochrome c oxidase-nitric oxide complexes. Biochim Biophys Acta 1982;679:84–94.
34. Boelens R, Wever R, Van Gelder BF, Rademaker H. An EPR study of the photodissociation reactions of oxidized cytochrome c oxidase-nitric oxide complexes. Biochim Biophys Acta 1983;724:176–183.
35. Gordon SA, Surrey K. Red and far-red light action on oxidative phosphorylation. Radiat Res 1960;12:325–339.
36. Kato M, Shinzawa K, Yoshikawa S. Cytochrome oxidase is a possible photoacceptor in mitochondria. Photobiochem Photobiophys 1981;2:263–269.
37. Passarella S, Casamassina F, Molinari S, Pastore D, Quagliariello E, Catalano Cingolani IM. Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by He–Ne laser. FEBS Lett 1984;175:95–99.
38. Yu W, Naim JO, McGówan H, Ippolito K, Lanzafame RJ. Photomodulation of oxidative metabolism and electron chain enzymes in rat liver mitochondria. Photochem Photobiol 1997;66:866–871.
39. Pastore D, Greco M, Passarella S. Specific He–Ne laser sensitivity of the purified cytochrome c oxidase. Int J Radiat Biol 2000;76:863–870.
40. Adar F, Yonetani T. Resonance raman spectra of cytochrome c oxidase evidence for photoreduction by laser photons in resonance with Soret band. Biochim Biophys Acta 1978;502: 80–86.
41. Hallen S, Oliveberg M, Brzezinsky P. Light-induced structural changes in cytochrome c oxidase. Measurement of electrogenetic events and absorbance changes. FEBS Lett 1993;318:134–138.
42. Hallen S, Brzezinski P. Light-induced structural changes in cytochrome c oxidase: implication for the mechanism of electron and proton gating. Biochem Biophys Acta 1994;1184: 207–218.
43. Zhang YO, Song SP, Fong CC, Tsang CH, Yang Z, Yang MS. cDNA microarray analysis of gene expression profiles in human fibroblast cells irradiated with red light. J Invest Dermatol 2003;120:849–857.