MULTIPLE LOW LEVEL LASER IRRADIATION EFFECTS ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND PLATELETS REVEALED BY FLUORIMETRIC TECHNIQUES

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Abstract. The aim of the present study was to supply new data concerning the cellular effects of low power long wavelength laser irradiation. Steady state fluorescence intensity measurements with the use of appropriate fluorophores allowed disclosure of changes induced by the 680 nm red and the 830 nm infrared laser radiation in the polar headgroup region lipid order parameter of plasma membranes as well as in the intracellular calcium levels of human platelets and peripheral blood lymphocytes. Comparison of the effects seen in freshly separated and in metabolically impaired cells revealed a sensitive modulation of laser irradiation effects by the actual metabolic state of the cells.

Key words: AlGaInP/GaAs laser, metabolic impairment, fluorescence anisotropy, TMA-DPH, cell calcium, Fura2-AM.

INTRODUCTION

Many physical factors of low intensity and/or chemicals in a low concentration region exert stimulatory or inhibitory effect on living systems, but in spite of extensive cellular studies, the underlying action mechanisms are largely unknown. Regardless of the irritant type, responses of the reacting systems (i.e. cells, tissues) have much in common, and are often described in terms of the adaptation syndrome [16]. It is also assumed that the biochemical basis of nonspecific responses of cells is a change in their energy metabolism [8]. Nonetheless, the detailed molecular and cellular mechanisms involved remain elusive. The aim of this study was to gather data concerning the effects on cell membrane properties and ion homeostasis of one such physical factor, namely the low-level laser radiation in the red and near infrared region, in freshly separated

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and in metabolically impaired human peripheral blood lymphocytes and platelets. Data obtained reveal membrane effects of low power 680 nm and 830 nm laser light in human blood cells *in vitro*, as well as laser induced changes in resting calcium levels, modulated by the actual metabolic state of the cells.

MATERIALS AND METHODS

Chemicals: The fluorescent lipid probe 1[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), and Fura2-acetoxymethylester (Fura2-AM) were from MOLECULAR PROBES, HEPES buffer substance from SIGMA CHEMICAL Co., while all other chemicals were the best research grade available.

Suspension media: HEPES buffer 1 (HPMI, constituted of 100 mM NaCl, 5.4 mM KCl, 0.4 mM $MgCl_2$, 0.04 mM $CaCl_2$, 10 mM Hepes, 20 mM glucose, 24 mM $NaHCO_3$ and 5 mM Na_2HPO_4, pH 7.4), and HEPES buffer 2 (constituted of 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, pH 7.4).

Cells: Human peripheral lymphocytes and platelets were obtained from venous blood collected in a citrate buffer, of drug-free healthy volunteer donors. All donors signed an informed consent, and protocols were approved by the University Ethics Committee. Lymphocytes were separated using the standard Ficoll-Hypaque density gradient sedimentation technique [15]. Platelets were prepared from platelet rich plasma by centrifugation/resuspension [3].

Metabolic impairment: cells were metabolically impaired by nutritional supplement (serum, amino acids or vitamins) starvation at temperatures below 37 °C. Human platelets and peripheral lymphocytes were kept various time periods (1–48 hours) at room temperature or at 4 °C, in glucose-containing HEPES buffers 2 and 1 respectively. Samples exposed to these conditions contain cells with intact, but depolarized plasma membranes (more than 88% trypan blue excluding, more than 25% depolarized, data not shown). We name “fresh”, samples constituted of suspensions of lymphocytes freshly separated within 90 min after blood drawing or of platelets freshly transferred from 1–4 h old platelet rich plasma in supplement-free buffer. These suspensions contain “metabolically intact” cells, having membrane potentials altered less than 20%, and viabilities higher than 92% (data not shown).

Samples: were constituted by continuously stirred, thermostatted cell suspensions prepared in HEPES buffers at concentrations of $10^5 – 2\times10^6$ cells ml^{-1} for lymphocytes, and $10^6 – 2\times10^7$ cells ml^{-1} for human platelets.

Labeling of cells with fluorescent probes: TMA-DPH labeling was performed by incubating the cells in the measuring cuvette with 1 μM dye at 37 °C for 3 min at the beginning of each individual experiment. For Fura-2 labeling, the peripheral blood lymphocytes were incubated with 1.2μM Fura2-AM for 45 min at 37 °C (2×10^6 cells ml^{-1}), pelleted, resuspended in HEPES buffer, and used immediately for intracellular calcium level measurements.

Steady state fluorescence intensity measurements were carried out using computer-connected spectrofluorimeters: JOBIN-YVON JY3, PERKIN-ELMER MPF 44B and AMINCO-BOWMAN 4-8202Ba, equipped with thermostatted cell holders, stirring devices, POLAROID HN polarizers, and dedicated data acquisition software.

Steady state fluorescence anisotropy values (r) were obtained by quasi-simultaneous (within 20 s) measurements of the intensity components I_{VV} and I_{VH}. VV and VH stand for vertical/vertical (parallel) and vertical/horizontal (perpendicular) positions of the excitation and emission polarizers respectively. A correction factor for unequal transmission of the two components, $G = I_{HV}/I_{HH}$, was also determined and fluorescence anisotropy [11, 12] was calculated as:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \tag{1}$$

Measurements were made within 60 min after dye loading. The small autofluorescence of cells was subtracted from each intensity component.

The lipid order parameters at the surface polar headgroup regions of the cells plasma membrane [13, 17] were calculated from fluorescence anisotropy values of the positively charged lipid probe TMA-DPH, measured at λ_{emm} = 425 nm (λ_{exc} = 340 nm):

$$S = \sqrt{r_\infty / r_0} \tag{2}$$

where the limiting initial value of the fluorescence anisotropy $r_0^{\text{TMA-DPH}} = 0.362$, while the limiting long-time values of the fluorescence anisotropy r_∞ were approximated using a formula based on published experimental data [1]:

$$r_\infty = 1.270 \times r - 0.076 \text{ for } 0.13 \le r \le 0.28 \text{ and} \tag{3}$$

$$r_\infty = 1.100 \times r - 0.032 \text{ for } 0.28 < r \le 0.34. \tag{3'}$$

Fura2 fluorescence was measured in ratio mode with emission wavelength of 510 nm and alternating excitation wavelengths of 340 nm/380 nm, and *cytoplasmic calcium concentration* $[Ca^{++}]_i$ values were calculated [4]:

$$[Ca^{++}]_i = K_D^{\text{Fura2}} \cdot \frac{R - R_{min}}{R_{max} - R} \tag{4}$$

where, $R = I_{340\text{ nm}}/I_{380\text{ nm}}$ represents the Fura2 fluorescence intensity ratio, R_{min} is the value of this ratio in calcium-free medium (6 mM EGTA, pH = 8), R_{max} the value measured in presence of calcium and of a plasma membrane permeabilizing agent (0.8 mM digitonin), while the value of K_D^{Fura2} is 224 nM at 37 °C.

The irradiation sources were AlGaInP/GaAs based semiconductor lasers used in the medical practice, PHILIPS CQL806D and SONY SLD202-D3, with emission wavelengths and nominal powers of 680 nm / 25 mW, and of 830 nm / 50 mW, respectively.

Sample irradiation was performed directly in the fluorimeter, with sources placed at a 6 cm height from the upper surface of cell suspensions, and the detector protected from any scattered laser light by a BG12 filter. Duration of treatment varied between 12–600 s, giving at the average incident power densities of approximately 234 Wm^{-2} and 286 Wm^{-2} for the red and infrared laser beam, respectively, total incident doses lying in the range of 2.8–172 kJm^{-2}.

Data Analysis: Anisotropy, lipid order parameter and intracellular calcium concentration values are presented as mean ± S.D. calculated from at least 3 independent measurements. Relative changes in these parameters under the influence of laser irradiation were calculated as ratios of differences between values measured in 3 consecutive moments and the initial control average value (as calculated from values measured in the first 3 consecutive moments) and the initial control average. Unpaired analysis of data series obtained by measurements made on cells various time periods after their transfer in serum-free buffer, before, during and after laser irradiation, was performed by Student's t-test (two-tailed). P-values less than 0.05 were regarded as statistically significant.

RESULTS

In our hand the average TMA-DPH fluorescence anisotropy values measured within the time period of 20–60 min after dye loading, at 37 °C, in human peripheral blood lymphocytes and platelets of healthy volunteers were 0.245 ± 0.011 and 0.256 ± 0.012, respectively. The corresponding values of the second rank order parameter of membrane lipids in the polar headgroup region on the inner surface of plasma membrane are 0.806 ± 0.009 and 0.830 ± 0.009, respectively. The intracellular calcium concentrations measured in human peripheral blood lymphocytes within the time period of 10–100 min after their resuspension in the 0.04 mM $CaCl_2$ containing medium were 80 ± 20 nM.

The data presented here document significant changes ($p \leq 0.01$ in at least 3 independent experiments) in the average lipid packing density in the polar headgroup regions of the human peripheral blood lymphocytes and platelets plasma membrane bilayer during and after multiple irradiation with red (680 nm) and/or infrared laser light (830 nm) (Figs. 1–3). The observed effects were considerably dependent on the actual metabolic state of the cells.

The changes observed were slowly reversible in time, immediately after irradiation, or even 5 min later, the calculated lipid order parameter values being still different from those characteristic for non-irradiated cells. The magnitude of the observed changes was dependent on the wavelength of laser radiation, order and frequency of irradiation, total dose, fluence rate, and the nature of the cells. Similarly, changes in intracellular calcium concentration were apparent ($p<0.05$) during and following 830 nm irradiation of human peripheral blood lymphocytes (Fig. 2).

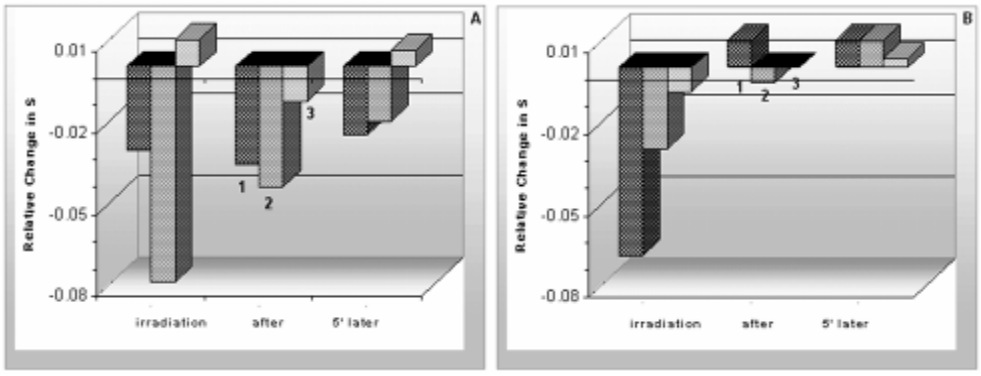

Fig. 1. Changes induced in the lipid order parameter S of human peripheral blood lymphocytes (A) and platelets (B) plasma membrane polar headgroup region during, immediately after, and 5 min after a single irradiation of a total dose of approximately 17 kJm^{-2} (234 Wm^{-2}, 76 s) with a 680 nm laser light; 1 – "fresh" samples (cells within 1 h after blood drawing), 2 and 3 – cells exposed to conditions leading to metabolic impairment 8 h and 24 h, respectively. Mean values calculated from data gathered in at least 3 independent experiments (S.D.≤ 12%).

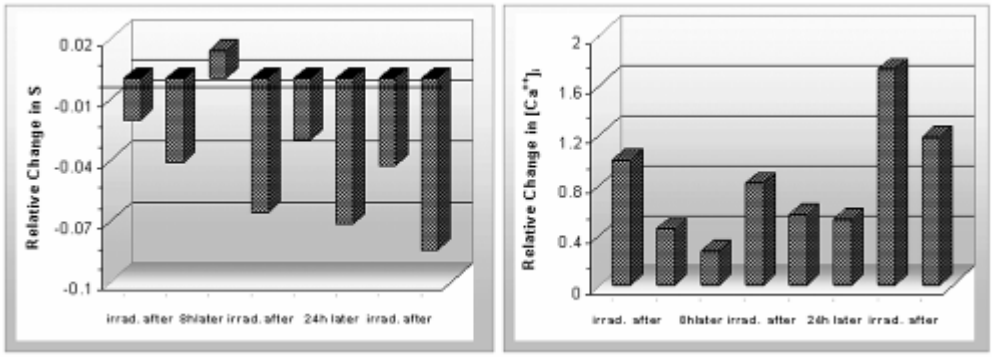

Fig. 2. Changes induced in plasma membrane lipid order parameter *S*, and in intracellular calcium concentration $[Ca^{++}]_i$ of human peripheral blood lymphocytes, by multiple irradiation with 830 nm laser light. Total dose in single irradiation: approximately 17 kJm^{-2} (286 Wm^{-2}, 60 s). Mean values calculated from data gathered in at least 3 independent experiments (S.D. ≤ 15%).

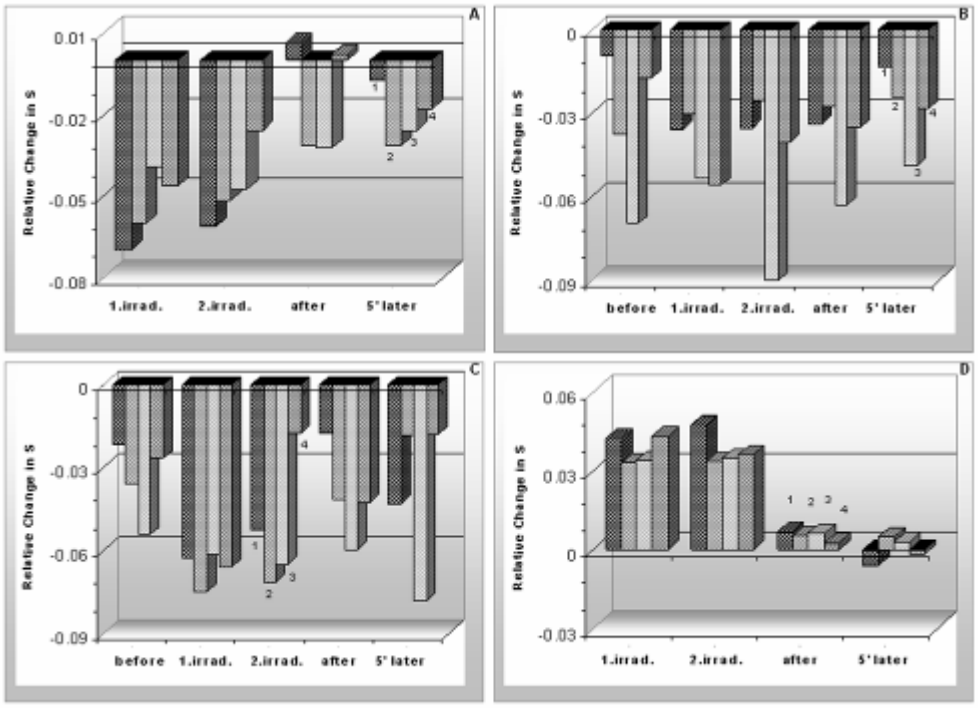

Fig. 3. Changes induced in the lipid order parameter S of human peripheral blood lymphocytes plasma membrane polar headgroup region by multiple irradiation with laser light of different wavelengths, within 50 min following cell separation from freshly drawn blood (A), 90 min later following the first irradiation (B), 60 min later following the second irradiation (C), and after 24 h rest following the third irradiation (D). Order of irradiation: 830 nm – 830 nm (1), 680 nm – 830 nm (2), 680 nm – 680 nm (3), 830 nm – 680 nm (4). Total dose in single irradiation: ~17 kJm^{-2} (234 Wm^{-2}, 76 s, and 286 Wm^{-2}, 60 s for the 680 nm and 830 nm laser light, respectively). Mean values calculated from data gathered in at least 3 independent experiments (S.D. ≤ 16%).

DISCUSSION AND CONCLUSIONS

The 680 nm and 830 nm lights are recognized to be absorbed by the oxidized forms of the so-called Cu_B and Cu_A redox active centers of the mammalian mitochondrial respiratory chain terminal enzyme cytochrome C oxidaze [2, 8] and they are supposed to further oxidize these centers, thus promoting enhancement of the electron transfer rate [8]. Absorbing entities in the plasma membrane of eukaryote cells are not known. Nonetheless, laser irradiation effects on cell adhesion were repeatedly evidenced [5, 6] and we also published fluorimetric data concerning fluidization of the inner surface of the plasma membrane of human blood cells under the influence of the 680 nm [9, 14] and the 830 nm [10] laser light. The TMA-DPH fluorescence anisotropy data presented here document once more significant changes in the average lipid packing density in the polar

headgroup region of both the single and the multiple irradiated human peripheral blood lymphocytes and platelets plasma membrane, both in case of monochromatic and of mixed (alternating red/infrared) irradiation regimes. Even though, the complex dependence of the effects on order, fluence rate, frequency and intensity of irradiation is not obvious, and needs further investigations (currently undertaken in our laboratories), modulation by the actual metabolic state of cells is apparent. At our best knowledge, our data are the first providing sound evidence for direct membrane effects of low power 680 nm and 830 nm laser light in human blood cells *in vitro*.

Short-term changes under the influence of 633 nm He-Ne laser radiation in the cytosolic calcium concentration in human peripheral blood lymphocytes were previously reported [7]. Our studies disclose qualitatively similar changes in cellular calcium levels in human peripheral blood lymphocytes irradiated with the 830 nm laser light. Though, the dependence on the actual metabolic state of the cells is evident, interpretation of the observed effects is not trivial. Studies concerning the nature and significance of the laser-induced calcium level changes and calcium signals in different cells are currently in progress in our laboratories.

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