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Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro

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Abstract

A monolayer of HeLa cells was irradiated with an He-Ne laser (632.8 nm, 100 J m^{-2}, 10 s) and the amount of adenosine triphosphate (ATP) was measured by the luceferin-luciferase bioluminescent assay technique at different times (5-45 min) after irradiation. The amount of ATP in the log phase of cultured cells remained at the control level ((0.79 + 0.09) x 10^{-15} mol per cell) during the first 15 min after irradiation; it then increased sharply and, after reaching a maximum (170.8%) 20 min after irradiation, decreased slowly to the control level. The ability of monochromatic red light to induce an increase in the cellular ATP level was found to depend on the growth phase of the culture, being insignificant in the lag phase of cultured cells, increasing in the log phase of cultured cells and reaching a maximum (about 190%) in c ells at the late logarithmic and early plateau phase.

Keywords: ATP; HeLa cells; Low-power laser therapy ; Mitochondria

1. Introduction

Several lines of evidence show that mitochondria are sensitive to irradiation with monochromatic visible light. The illumination of isolated rat liver mitochondria increases adenosine triphosphate (ATP) synthesis and the consumption of O_2 [1-3]. Irradiation with light at wavelengths of 415 nm [I], 602 nm [4], 633 nm [2], 650 nm [3] and 725 nm [3] enhances ATP synthesis. Light at wavelengths of 477, 511 and 554 nm [1] does not influence the rate of this process. Oxygen consumption is activated on illumination with light at 365 and 436 nm, but not at 313, 546 and 577 nm [4]. Irradiation with light at 633 nm increases the mito-chondrial membrane potential ($\Delta\psi$) and proton gradient (ΔpH) [2], causes changes in mitochondrial optical properties, modifies some NADH-linked (NADH, reduced form of nicotinamide adenine dinucleotide) de-hydrogenase reactions [5] and increases the rate of ADP/ATP exchange (ADP, adenosine diphosphate) [6].

Investigations into the Oд consumption and ATP content of whole cells (not isolated mitochondria) have received much less attention. Irradiation of yeast cells at λ ==633 nm activates Oz consumption and increases the activity of NADH dehydrogenase and cytochrome c oxidase [7,8]. An increase in ATP synthesis of R3230AC mammary adenocarcinoma cells after irradiation at λ =630 nm was observed in "light only" control experiments during an investigation of the mechanism of photodynamic action of certain chemicals [9]. Irradiation of human peripheral lymphocytes with light at 820 nm increases the amount of ATP in these cells [10].

The aim of the experiments reported in this paper was to measure the ATP level in preliferating and non-proliferating HeLa cells. Initially, the ATP levels were measured in culture at different times following exposure to 632.8 nm light. The amounts of ATP synthesized by cells in different cell culture growth phases were compared. The experiments were performed with HeLa cells, i.e. on the same model as used previously for the investigation of the activation of light-induced proliferation [II].

2. Materials and methods

2.1. Cells

HeLa cells, initially obtained from the Institute of Virology, Moscow, Russia, were grown as monolayers in scintillation vials in medium 199 with the addition

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of 10% bovine serum and 100 units ml^{-1} penicillin and streptomycin. The cultivation details and growth characteristics of the culture are described elsewhere [II].

2.2. *Irradiation*

For irradiation, an He-Ne laser (λ = 632.8 nm, Spectra Physics model 125A) was used. The beam was expanded with a short-focus positive lens to a diameter of 24 mm which corresponded to the diameter of the bottom of the vial covered with the monolayer (Fig. 1). Neutral filters were used to reduce the intensity of light to 10 W m^{-2}. The irradiation time was 10 s, and the fluence was 100 J m^{-2}. These parameters have been found to be optimal for the activation of proliferation of HeLa cells grown in a monolayer [II]. A Spectra Physics M404 power meter was used for measurement of the light intensity. The vial was placed into a special holder and remained closed during irradiation. The monolayer was not covered with the medium due to the position of the vial (Fig. 1). Irradiation was performed in the dark through the bottom of the vial. The control samples were treated in exactly the same way but were not irradiated.

2.3. Measurement of ATP level in cells

The amount of ATP was determined by bioluminescent assay based on the measurement of-the light output of the luciferin-luciferase reaction [12]. The luciferin-luciferase was purchased as a kit from LKB-Wallac (Turku, Finland). The ATP mounting kit also contained *N*-2-hydroxyethylpiperazine-*N*' -2-ethanesul-phonic acid (HEPES) buffer, trichloroacetic acid and ATP standards. The luminescence of the samples was measured with a Biolumat LB 9500 (Berthold, Germany). The amount of ATP was determined by calibration using the ATP standards.

At definite times after irradiation (5-45 min), the nutrient medium was removed, the monolayer was rinsed with ice-cold Hanks' balanced salt solution and kept for 5 min on ice with 0.5 mM of 7% trichloroacetic acid (containing 10 mM ethylenediaminetetraacetic acid (EDTA)) without shaking. The sample was diluted with HEPES buffer (pH 7.75); a 200 μl sample solution was transferred to a Biolumat test vial containing 20 μl luciferin-luciferase and the luminescence was measured during 10 s. All procedures were performed in the dark. The number of cells was counted by a haemocytometer and the data were expressed as the number of moles of ATP per cell (mean ± standard error of the mean (SEM)) for at least three separate vials per point per experiment.

3. Results

In the first series of experiments, the amount of ATP was measured in the log phase of cultured cells (72 h after plating) as a function of the time elapsed after irradiation of the cellular monolayer with an He-Ne laser (100 J m"^{2}, 10 s). The amount of ATP per individual cell in the controls was (0.79 ± 0.09) x 10^{-15} mol per cell. As can be seen from Fig. 2, the amount of ATP (expressed as a percentage of the above control value) remains practically at the control level for several minutes after irradiation. An insignificant increase is observed for the first 15 min after irradiation, followed by a strong increase over the next few minutes. After a maximum is reached (170.8%, 20 min after irradiation), the ATP level decreases slowly to the control level.

In the next series of experiments, we investigated the dependence of the increase in the ATP level on the growth phase of the culture. To this end, the same experiment was performed on different days after plating: the monolayer was irradiated for 10 s (100 J m^{-2}),

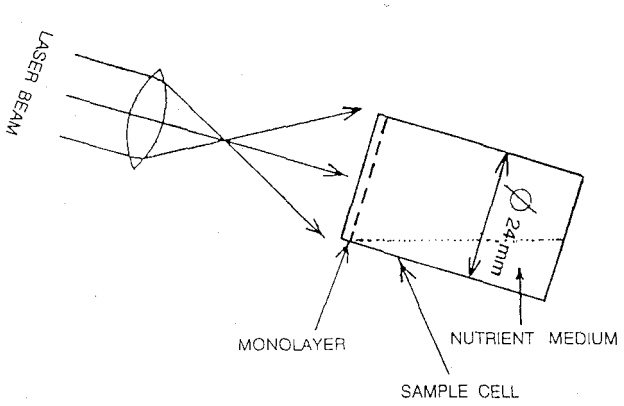

Fig. 1. Diagram of the irradiation of the cellular monolayer.

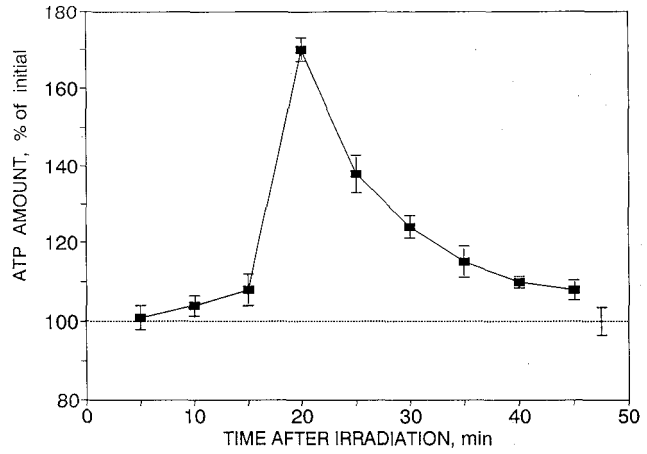

Fig. 2. ATP level in HeLa cells during the exponential phase of growth (72 h post-plating) as a function of the time elapsed after irradiation (632.8 nm, 100 J m^{-2}, 10 s).

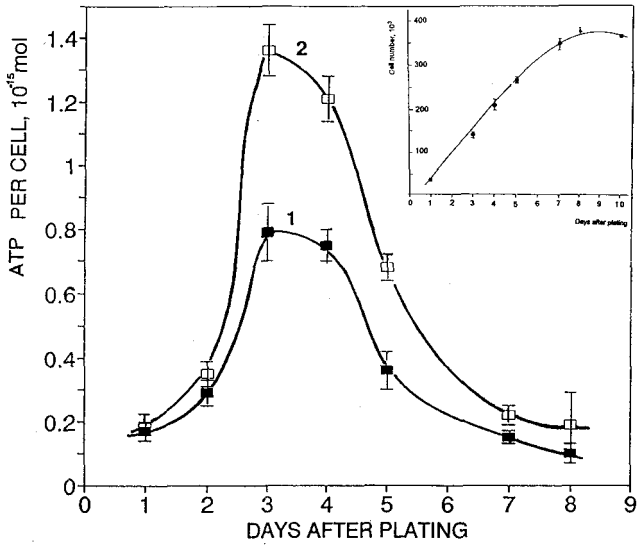

Fig. 3. Amount of ATP in cells at various growth phases: (1) non-irradiated cells; (2) irradiated cells. The amount of ATP per cell was measured 20 min after irradiation (632.8 nm, 100 J m^{-2}, 10 s). The inset depicts the growth curve of non-irradiated cells.

the amount of ATP was measured 20 min after exposure and this value was compared with the amount of ATP in the non-irradiated cells. The results of these measurements are presented in Fig. 3. It can be seen from Fig. 3 (curve 1) that the amount of ATP in non-irradiated cells remains low during the lag phase (0-24 h after plating, see the growth curve in the inset of Fig. 3) and at the beginning of the log phase (24-48 h post-plating). In the middle of the log phase (72 h post-plating), the amount of ATP reaches a maximum ((0.79 ± 0.09) x 10^{-15} mol per cell) and decreases at the onset of the plateau phase (7-8 days after plating). This change in the ATP content of the cells is consistent with literature data [13].

Curve 2 in Fig. 3 presents the ATP data in irradiated cells. A comparison between curves 1 and 2 of Fig. 3 enables the following conclusions to be drawn. In the cells in the lag phase and at the beginning of the log phase (1-2 days after plating), the photoinduced increase in the amount of ATP per cell is insignificant. ATP levels are enhanced in irradiated cells in the log phase (3-5 days) and plateau phase (8th day after plating) of growth. Thus the maximal ATP level induced in cells by irradiation is dependent on the growth phase of the culture.

4. Discussion

The data obtained show that the irradiation (Л = 632.8 nm) of cells cultivated in vitro increases the amount of ATP. This result is not surprising, given the results on light-induced ATP extrasynthesis in isolated mitochondria [1-6]. More unexpected is the shape of the corresponding curve (Fig. 2): the cellular ATP content does not change during the first 15 min after irradiation and increases significantly only 20-25 min after irradiation. The results of experiments on isolated liver mitochondria clearly show that both O_2 consumption and ATP extrasynthesis start as soon as the illumination is switched on and stop when the light is switched off [1,4]. Some experimental results show that ATP synthesis in irradiated cells may continue at enhanced rates after the illumination is switched off. This suggestion is based on the following experimental data. After a period of 18 h after the irradiation of yeast cells at 632.8 nm for a short time (a few minutes), the activities of the mitochondrial enzymes NADH dehydrogenase and cytochrome c oxidase were 241% and 128% compared with that in control cells (100%) [7]. Electron microscopy investigations demonstrate that similar changes occur in isolated mitochondria [14,15], human lymphocytes [16,17] and presynaptic terminals of the dorsal horn of cats [18] after irradiation with red light at 632.8 nm. The reconstruction of mitochondria from ultrathin sections through a whole lymphocyte has shown that, 1 h after irradiation with an He-Ne laser (for 10 s), the number of mitochondria is reduced to 9-12 compared with 40-45 in control cells; 2-4 branching giant mitochondria were also obtained [17]. Electron microscopy did not reveal any changes in the mitochondria of irradiated cells which might be considered degenerative. In contrast, within the framework of Skulachev's theory of a power-transmitting proton cable, the formation of giant mitochondria provides a higher level of respiration and energy turnover [17]. Irradiation with an He-Ne laser results in an increase in both mitochondrial RNA and protein synthesis [19]. It was concluded in Ref. [19] that these findings are consistent with a significant modification of the mitochondrial synthetic apparatus rather than minor changes in enzymatic activity. The data presented in Refs. [14-19] suggest that ATP synthesis in whole cells does not stop when the illumination is switched off.

Usually the energy charge of cells in culture is approximately 0.9 or more [20], indicating that more than 80% of the adenine nucleotides will be ATP. If this is the case under the present conditions, the increase in the cellular ATP level induced by 632.8 nm light must be due to the synthesis of new adenine nucleotides and the phosphorylation of ADP and adenosine monophosphate (AMP). The synthesis of new adenine nucleotide molecules may explain the delayed increase in cellular ATP. These possibilities may be distinguished by measuring ADP and AMP which was not carried out in this work.

The mechanism involved in the increase in the amount of ATP in cells irradiated with visible light is unknown. At least four possibilities have been discussed in recent

years. Fig. 4 summarizes these mechanisms. This is merely an illustrative scheme indicating the relevant reactions that occur after electronic excitation of photoacceptor molecules (cyt a/a_3 and NADH hydrogenase [21]). It does not mean that all of these reactions occur from the first singlet state and that triplets are not involved. Experimental studies on the primary mechanism are few [22]. In Ref. [22], it was shown that, on illumination in the presence of riboflavin, ATP synthesis occurs from ADP and P. This confirms the dual role of ADP (both electron donor and acceptor) in the photoreaction with riboflavin.

Fig. 4 presents several possible reactions. Firstly, on the basis of the monochromatic visible light action spectra for DNA and RNA synthesis in HeLa cells, and the spectroscopic data for porphyrins and flavins (endogenous photosensitizers), a hypothesis was advanced that the absorption of light quanta by these molecules is responsible for the generation of singlet oxygen $^1O_2$ [23a,b]. Photodynamic reactions have been considered for some time as the predominant reactions during the irradiation of cells at high doses and intensities of light [21]. The possible role of singlet oxygen in low-power laser experiments has been discussed by several groups [24,25]. However, no experiments demonstrating the generation of $^1O_2$ in cells or tissues after irradiation with low-power laser light have been observed.

Another process involves the acceleration of electron transfer in the respiratory chain due to a change in the redox properties of the carriers following photoexcitation of their electronic states [26]. A similar principle governs the functioning of photosynthetic reaction centres.

During light excitation of electronic states, a notable fraction of the excitation energy is converted to heat, which causes a local increase in the temperature of the absorbing chromophores [27]. The local transient rise in the temperature of photoacceptor molecules, as a possible mechanism of the low-power laser effect, is considered in Ref. [28]. It should be noted that the local transient heating of absorbing molecules is very different from average heating of the whole cell, which is certainly not observed at the doses and intensities used in experiments with cells [21,28].

It is well known that, in mitochondrial electron transport, the superoxide radical $O_2^{\bullet -}$ is produced, and its rate of production primarily depends on the metabolic state of the mitochondria [29]. By activation of the electron flow in the respiratory chain of irradiated cells, we can expect an increase in $O_2^{\bullet -}$ production. It has been demonstrated experimentally that mitochondria possess a mechanism for the reabsorption of $O_2^{\bullet -}$, and $O_2^{\bullet -}$ may be a source of electrons for the oxidative phosphorylation of ADP under physiological conditions [30]. Experimental data show that liver mitochondrial ATP synthesis can be inhibited and promoted by the UV generation of $O_2^{\bullet -}$ [31].

There is no reason to believe that only one of the processes discussed above (Fig. 4) occurs when a cell is irradiated. Recently, the existence of more than one reaction channel for the same final photobiological effect (growth stimulation of *Escherichia coli)* has been shown [28]. The question seems to be which channel is responsible for the photobiological effect observed in this study.

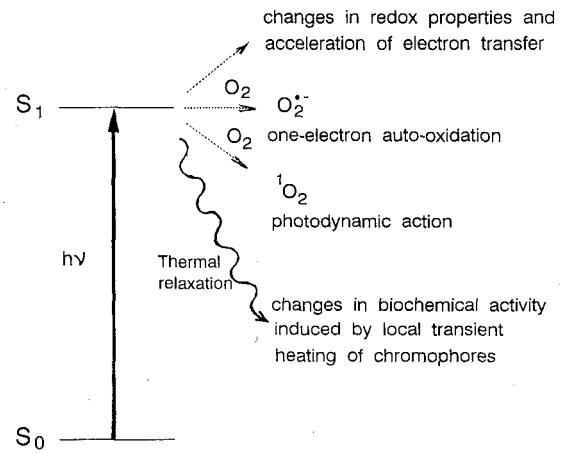

Fig. 4. Diagram indicating the possible reactions with photoacceptor molecules (respiratory chain components) after promotion to excited electronic states (for details, see text).

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