Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by helium-neon laser

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1. INTRODUCTION

The increasingly wide use of He-Ne laser in phototherapy has led to greater interest in the mechanism of He-Ne laser–biosystem interactions.

He-Ne laser irradiation has recently been reported to change the optical and biochemical properties of both NADH and RNA [1–4], while it has been shown that laser irradiation neither damages mitochondrial structure nor affects mitochondrial enzyme compartmentation and membrane permeability. Laser irradiation has also been found to influence the activity of some NADH-linked mitochondrial reactions in isolated liver mitochondria in vitro [4,5], although to date no information is available on the effect of laser irradiation on main mitochondrial functions, i.e., energy transduction, ATP synthesis and cellular respiration.

We report initial studies on the effect of He-Ne laser irradiation on mitochondrial energy metabolism. He-Ne laser irradiation is shown to generate an extra electrochemical potential as well as to cause an increase in ATP synthesis.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinopropanesulphonic acid; N-RLM, non-irradiated rat liver mitochondria; L-RLM, irradiated rat liver mitochondria

2. MATERIALS AND METHODS

All reagents used were purchased from Sigma except FCCP, which was kindly supplied by Dr P. Heytler (du Pont).

As previously reported [4], mitochondria were isolated from male Wistar rats (200–250 g) fed ad libitum.

Mitochondrial irradiation: in all cases a single mode continuous wave He-Ne laser (wavelength 6328 Å) was used, whose most significant features have previously been described [1,4]. Irradiation was carried out at a power of ~15 mW to give an energy dose of 5 J/cm^2.

In the case of both oxygen uptake and 'ΔpH' measurement, two aliquots (1 ml) of freshly prepared mitochondria (~50 mg protein) were put in quartz cuvettes in a suitable holder kept at 0°C by an ice-water mixture, one irradiated and the other used as a control.

In the case of both '$\Delta\psi$' and ATP content

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measurements, mitochondria were diluted immediately prior to each assay with the reaction medium to give suspensions containing 1 and 2 mg protein/ml respectively. Two 1-ml aliquots were taken from these suspensions, the first irradiated as previously described, and the second used as a control, kept under the same conditions with no irradiation.

'$\Delta\psi$' changes were monitored by means of safranine according to [6] using a Beckman DU-7 HS spectrophotometer. Spectra were carried out at 20°C in 2 ml of a standard medium (0.25 M sucrose, 20 mM Tris-HCl, pH 7.25, 1 mM EGTA-Tris) containing 12.5 μM safranine and 0.5 mg mitochondrial protein/ml. The preparation of each test involved adding 1 ml of safranine dissolved in the reaction medium (25 μM) to the 1-ml aliquots of both irradiated mitochondria and control prepared as described above. Other additions are described in the legends of the figures.

"ΔH' measurements were carried out according to [7], except that [C]-acetate was used. Measurements were made at 20°C in 1 ml of either a KCl medium of 0.15 M KCl, 20 mM MOPS-Tris (pH 7.2) or the standard medium. In both cases 1 μg rotenone, 1 μg antimycin A and 2 μg oligomycin were also present.

ATP measurements: mitochondrial ATP content was measured continuously at 27°C by luminescence of the firefly luciferase reaction according to [8] by means of a Perkin-Elmer LS-5 luminometer equipped with a biochemiluminescence accessory (cod. 5212 4999). Each sample was vigorously stirred. The assay suspension (2 ml) had a final composition of 0.15 mM sucrose, 0.5 mM EDTA, 5 mM $MgCl_2$, 7.5 mM Na_2HPO_4, 2 mM HEPES (pH 7.4) and 1 mg mitochondrial protein/ml, achieved with 1 ml of diluted mitochondrial suspension prepared as described above plus 1 ml of reaction medium added after irradiation. A detailed account of the method used to quantify the continuous luminescence signal is provided elsewhere [8].

Oxygen uptake measurements were followed by means of a Gilson 6H/5 oxygraph at 25°C, using a Clark electrode in 1.5 ml of the standard medium.

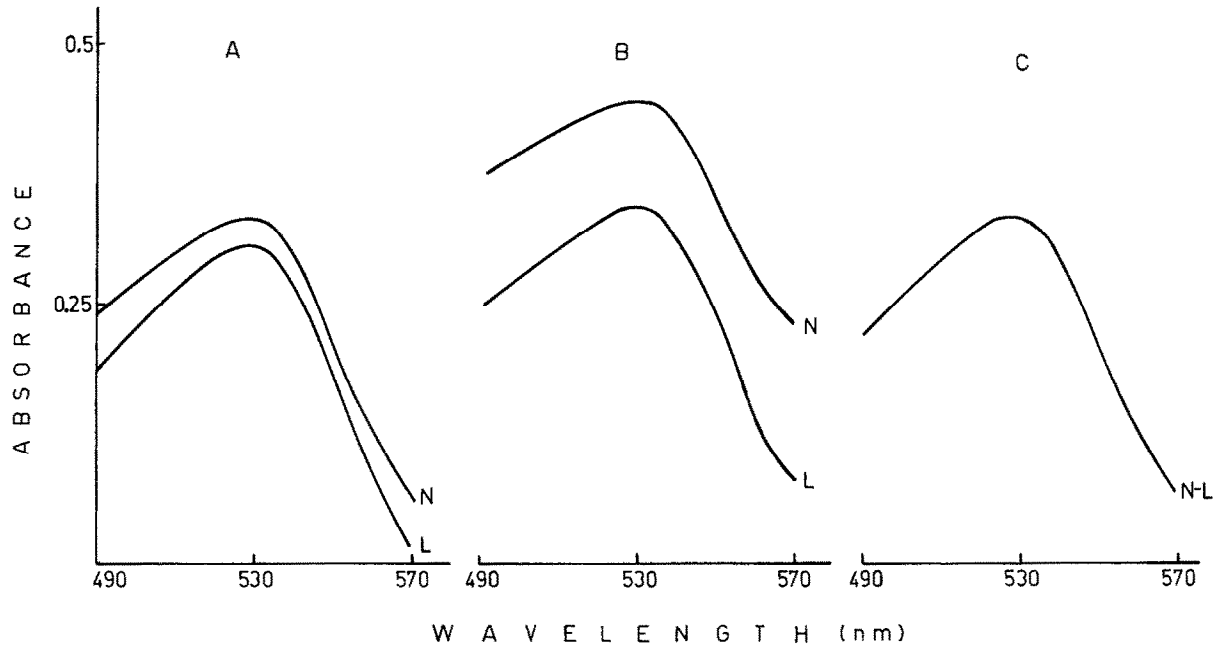

Fig. 1. Spectral changes in safranine following irradiation of mitochondria. Spectra were carried out as described in section 2. N-RLM (N) or L-RLM (L) were used. In B and C, oligomycin (2 μg) and rotenone plus antimycin A (2 μg each) respectively were added to the mitochondria (1 mg protein). Irradiation was carried out in the presence of these compounds.

The complete overlapping of experimental traces and values obtained with different mitochondrial samples assured the maximum reproducibility of each determination.

3.RESULTS

According to the Mitchell hypothesis a protonmotive force of two components, 'ΔpH' and '$\Delta\psi$', generated by biological oxidations, drives ATP synthesis in mitochondria. Possible changes in mitochondrial '$\Delta\psi$' and 'ΔpH' owing to laser irradiation were investigated. In the first case, use was made of the safranine O dye, which allows rapid measurement of transmembrane '$\Delta\psi$' while continuously monitoring changes in the membrane potential as a response to various disturbances [9]. A low power He-Ne laser was used to irradiate mitochondria. Irradiation (initial power about 15 mW, energy dose 5 J/cm^2) was carried out (i) in the absence of any inhibitor, (ii) in the presence of oligomycin and (iii) in the presence of rotetone and antimycin A.

Evidently laser irradiation increases mitochondrial membrane potential, as revealed by the decrease in safranine absorbance in L-RLM with respect to N-RLM along the entire wavelength investigated (fig. 1A). If oligomycin, which per se increases the absorbance of safranine in the presence of mitochondria (see also [10]), is added to both L-RLM and N-RLM, it causes a marked increase in their absorbance difference (fig. 1B) compared to that found in the absence of the compound (fig. 1A). On the other hand, the presence of rotenone and antimycin A completely prevents the change of safranine response caused by laser irradiation (fig. 1C).

To check possible changes in proton gradient caused by He-Ne laser irradiation, 'ΔpH' was measured in both N-RLM and L-RLM according to [7]. Increase in transmembrane proton gradient was found to be due to irradiation. In a KCl medium 'ΔpH' was found to be 0.286 and 0.336 in N-RLM and L-RLM, respectively. Moreover, some more changes were found when 'ΔpH' was measured in a low ionic strength sucrose medium: the proton gradient difference found in L-RLM compared to N-RLM ranged from 0.2 to 0.16 in several experiments.

Addition of the uncoupler FCCP in the assay medium after laser irradiation abolishes any difference between L-RLM and N-RLM in both transmembrane proton gradient and membrane potential (not shown).

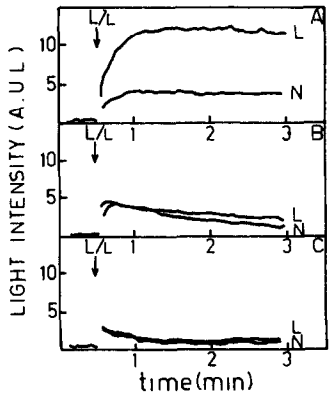

Fig. 2. Luminescence recordings of oxidative phosphorilation in L-RLM and N-RLM under different metabolic conditions. The assay was carried out as described in section 2. The reaction was started by addition of 50 μl of a solution containing 20mg/ml of the luciferase–luciferin reagent (L/L). In B and C, oligomycin (4 μg) and rotenone plus antimycin (4 μg each) were added to the mitochondria (2 mg protein). Irradiation was carried out in the presence of these compounds. ATP content in A, calculated according to [8], was 1.4 nmol/mg protein and 2.25 nmol/mg protein in N-RLM (N) and L-RLM (L), respectively.

Since irradiation basically causes an increase in the protonmotive force, investigation of a possible increase of ATP synthesis seemed worthwhile. Direct measurements of ATP production in L-RLM and N-RLM in the resting state were thus carried out (fig. 2). Mitochondrial ATP was measured by continuously monitoring ATP production by firefly luciferase luminescence according to [8]. An increase of mitochondrial ATP content was found in irradiated mitochondria when compared to control (fig. 2A). The extra ATP synthesis due to laser irradiation was found to be 0.85 nmol/mg protein with an increase of 70% compared to control content. Increase in ATP content was also confirmed by measurements on perchloric extracts of mitochondrial samples. In the same experiment ATP production was measured in mitochondria irradiated in the presence of either oligomycin (fig. 2B) or rotenone and antimycin A (fig. 2C). In these cases, although ex-

perimental traces did show a decrease in ATP concentration, no difference in ATP content was found between L-RLM and N-RLM.

The possible influence of laser-induced changes in electrochemical potential and ATP synthesis on the ability of mitochondria to take up oxygen was tested using succinate as substrate. The experimental data are reported in fig. 3, in which the dependence of the oxygen uptake rate on increasing succinate concentration was investigated as double-reciprocal plots with both N-RLM and L-RLM mitochondria. L-RLM show a reduced rate of succinate oxidation stimulated by ADP and P_i (state 3). Inhibition seems not to be competitive, as shown by the decrease of both V_{max} and apparent K_m values. This type of inhibition was confirmed by replotting the experimental data according to

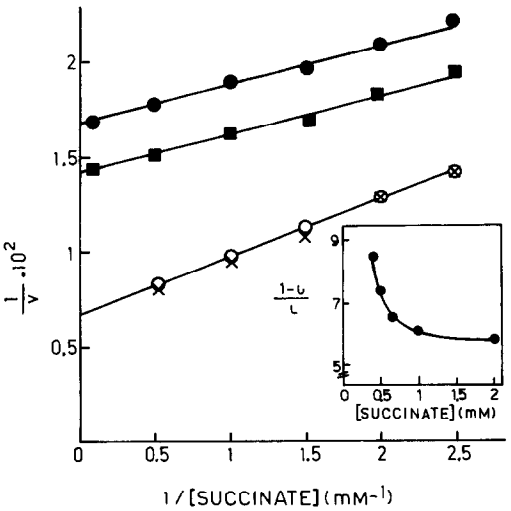

Fig. 3. Dependence of the rate of oxygen uptake by N-RLM and L-RLM on increasing succinate concentrations. The assay medium contained 0.25 M sucrose, 20 mM Tris-HCl (pH 7.25), 1 mM EDTA, 2 μg rotenone, at 25°C. Succinate oxidation was stimulated by the addition of either 1 mM ADP-Tris plus 1 mM P_i-Tris to both (■) N-RLM and (●) L-RLM, or of 1 μM FCCP to both (×) N-RLM and (○) L-RLM. In the inset the same data are replotted as advised in [11]. Owing to the impossibility of calculating the inhibitor concentration, $(1-i)/i$ is plotted instead of $[I](1-i)/i$ against the succinate concentration. Fractional inhibition, i, is equal to $(1-V_i)/V$, where V and V_i (expressed as natoms O_2/min × mg protein) are reaction rates in the presence of N-RLM and L-RLM, respectively.

[11] (inset, fig. 3). Incompetitive inhibition of ADP-stimulated succinate oxidation was also found in the presence of 1 μM ATP (not shown).

No apparent difference in V_{max} and K_m values was found between L-RLM and N-RLM when oxygen uptake was stimulated by the uncoupler FCCP.

4. DISCUSSION

This paper clearly shows that the irradiation of isolated rat liver mitochondria in vitro by low power He-Ne laser causes an increase in membrane potential, proton gradient and ATP synthesis.

This event appears to be strictly correlated with the mitochondrial electron transfer chain, as demonstrated by the complete lack of effects if laser irradiation is carried out in the presence of the inhibitors rotenone and antimycin A. The possibility of radiation-induced electron transfer giving rise to proton translocation cannot actually be excluded, largely in view of changes in the redox state of mitochondrial cytochromes due to laser irradiation (not shown). We suggest that extra ATP synthesis is directly produced by the laser-induced extra protonmotive force. In the presence of oligomycin, which completely blocks ATP synthesis and reduces oxygen uptake without directly interfering with the electron carriers in the mitochondrial inner membrane, irradiation causes a larger increase of membrane potential with no extra ATP synthesis. This could depend on the decreasing effect of the antibiotic on the potential under the conditions used (by abolition of the potential generated by endogenous ATP). However, the comparison between figs 1B and 2B suggests that it is related to lack of ATP synthesis in the presence of oligomycin.

These findings are consistent with the hypothesis that laser irradiation is absorbed by a putative photosensitizer in the mitochondrial compartments (see also [4]). Thus, an extra electrochemical potential, generated through a mechanism presently unknown, is subsequently discharged in ATP synthesis.

No inhibition of the mitochondrial respiratory chain by laser irradiation occurs, as shown by the lack of laser influence on FCCP-stimulated succinate oxidation. Thus, the reduced ability of L-RLM to consume oxygen stimulated by ADP + P_i

may be due to the increase of intramitochondrial ATP. In fact, under the same experimental conditions, the addition of 1 μM ATP has been shown to result in the incompetitive inhibition of oxygen uptake.

Finally, He-Ne laser light has been found to influence mitochondrial bioenergetics by giving extra ATP synthesis. Consistent with these results, ATP synthesis in intact cells irradiated by high power laser has been previously proposed [12]. Thus if further insight into this field is to be gained, different laser sources will have to be used under various experimental conditions, testing the specificity of coherent light as well.

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