Free Radical Research, Volume 38 Number 9 (September 2004), pp. 893–902

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ESR Detection of $^1O_2$ Reveals Enhanced Redox Activity in Illuminated Cell Cultures*

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Accepted by Professor H. Sies

(Received 25 March 2003; In revised form 10 July 2003)

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Low-energy visible light (LEVL) has previously been found to modulate various processes in different biological systems. One explanation for the stimulatory effect of LEVL is light-induced reactive oxygen species formation. In the present study, both sperm and skin cells were illuminated with LEVL and were found to generate singlet oxygen ($^1O_2$). The detection of $^1O_2$ was performed using a trapping probe, 2,2,6,6-tetramethyl-4-piperidone, coupled with electron paramagnetic resonance spectroscopy. In addition, we have shown that, together with $^1O_2$ generation, LEVL illumination increases the reductive capacity of the cells, which explains the difficulties encountered in $^1O_2$ detection. The potential of visible light to change the cellular redox state may explain the recently observed biostimulative effects exerted by LEVL.

Keywords: Singlet oxygen; Photobiostimulation; Light; EPR; TEMP

Abbrevations: LEVL, low energy visible light; ROS, reactive oxygen species; TEMP, 2,2,6,6-tetramethyl-4-piperidone; EPR, electron paramagnetic resonance; TEMPO, 2,2,6,6-tetramethyl piperidine-*N*-oxyl; 4-*O*-TEMPO, 4-oxo-2,2,6,6-tetramethyl-piperidine-*N*-oxyl; 4-*O*-TEMPO-H, 1-hydroxyl-4-oxo-2,2,6,6-tetramethylpiperidine; AFR, ascorbyl free radical

INTRODUCTION

Singlet oxygen at ($^1\Delta_g$) is a highly reactive species which can be generated in the living system by stimulated phagocytes during oxidative burst[1] or photochemically, as a result of endogenous or exogenous photosensitizers illuminated with visible or UV light.[2] Although $^1O_2$ is considered a deleterious species because it impairs biomolecules which in turn exert genotoxic and cytotoxic effects,[3,4] there is increasing evidence which shows that $^1O_2$ is involved in the activation of gene expression and intracellular signaling pathways, thus leading to cell biostimulation.[5–7]

One of the ways to induce cell biostimulation is by light. Recently, many studies have demonstrated the positive effects of visible light on various cells. For example, Passarella *et al*.[8] found that visible light elevates the mitochondrial membrane potential and increases ATP synthesis in isolated mitochondria. Yu *et al*.[9] demonstrated that 660 nm laser illumination stimulates the release of transforming growth factor β and platelet-derived growth factor from cultured fibroblasts. In our laboratory, we have demonstrated that HeNe laser light accelerates the cell mitosis of fibroblasts and keratinocytes.[10] The most important studies on light-induced responses of various eukaryotic and prokaryotic cells were performed by Karu and are summarized in her book.[11] Although the mechanism of light-induced cell activation is not sufficiently clear, it is hypothesized that $^1O_2$ is involved in this light-enhanced biostimulation.[12–14]

*The article describes research performed by Ronit Lavi as partial fulfillment of the requirements for her Ph.D. degree at Bar-Ilan University.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/1071576010001642646

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Unfortunately, the detection of $^1O_2$ in biological systems is not simple because of its short lifetime. For example, in cell-free aqueous systems, $^1O_2$ has a lifetime of 3–4 μs,[15] which decreases to about 200 ns in cellular systems. The rapid decay of $^1O_2$ due to cellular scavengers, such as reactive amino acids, limits its detection in biological systems.[16]

The most common method for direct detection of $^1O_2$ is by its IR emissions at 1269 and 1588 nm which accompany its relaxation to the ground state.[17,18] Kanofsky *et al.*[19] measured $^1O_2$ chemiluminescence at 1268 nm, which was emitted by human eosinophils stimulated by phorbol myristate acetate. Bilski *et al.* detected $^1O_2$ formation by its chemiluminescence (1200–1350 nm) in photosensitized cultures.[20,21] Nevertheless, because the high rate of destruction causes a substantial decrease in the steady state concentration of $^1O_2$, the intensity of the IR emission signal is too small to be detected easily in such a system. An indirect way of measuring $^1O_2$ is by a fluorescent probe molecule, such as 9,10-dimethylanthracene (DMA). When $^1O_2$ reacts with DMA, a decrease in DMA fluorescence is measured. Ehrenberg used this method to determine the generation rate of $^1O_2$ in photosensitized liposomes.[22] Another method is a trapping probe coupled with ^{1}EPR spectroscopy, which is also used in $^1O_2$ detection. A diamagnetic compound reacts with $^1O_2$, yielding a long-lived radical (spin adduct radical) detectable by electron paramagnetic resonance (EPR) spectroscopy.[23] A common trap for $^1O_2$ detection is 2,2,6,6-tetramethylpiperidine and its derivatives.[24–26] No EPR evidence for $^1O_2$ generation in illuminated cell cultures has yet been reported. The reasons for this are: (a) the high dielectric constant of water which absorbs the microwave energy during the EPR experiment and (b) the reducing capacity of the cells, especially under illumination (as shown in this work), which tends to quench the spin adduct radicals that are produced. Thus, the EPR coupled with a trapping technique for $^1O_2$ measurement has been used only for cell-free systems.[27–29] One exception is a recent work in which $^1O_2$ has been detected in the blood of mice stimulated by Cr(IV).[30]

In the present study, we have succeeded in trapping and detecting $^1O_2$ in illuminated fibroblasts and sperm cells using a gas-permeable Teflon capillary as the EPR sample compartment.[31]

MATERIALS AND METHODS

Illumination

We used a conventional source of visible light (400–800 nm), producing a power density of 80 mW/cm^2, to illuminate the cells. The EPR cavity grid transmits 50% of the light energy to the cells, such that illumination and measurement of the cells for a 3 min period caused the cells to be exposed to 7.2 J/cm^2 of energy. The incident light produced no detectable heating.

Cell Line

Fibroblasts

The NIH/3T3 fibroblastic (non-pigmented) cell line was used in this study. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l D-glucose and 2 mM L-glutamine supplemented with 10% newborn calf serum. The NIH fibroblasts were harvested and washed thrice by centrifugation at 500*g* for 10 min at room temperature. The final pellet of fibroblasts was resuspended in phosphate-buffer saline (PBS) and diluted to a final concentration of 5×10^7 cells/ml.

Fibroblasts Enriched with Hematoporphyrin

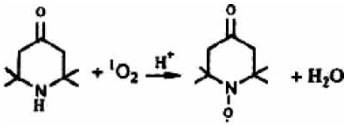
A measured quantity of 100 μg/ml hematoporphyrin (Sigma) was added to fibroblasts in the dark. After 1 h of incubation under standard conditions (37°C with 5% CO_2), the cultures were washed with PBS and irradiated as described.

Sperm Cells

Fresh ram semen was collected using an artificial vagina. The cells were then diluted (1:1) with buffer [NaCl, KCl, and MOPS (NKM)] containing 110 mM NaCl, 5 mM KCl and 10 mM MOPS (pH 7.4). The sperm cells were washed thrice by centrifugation at 780*g* for 10 min at room temperature. The final cell pellet was re-suspended in NKM buffer with 10 mM glucose to a final concentration of 10^9 cells/ml.

EPR Measurements

For detection of $^1O_2$, we used the trap probe 2,2,6,6-tetramethyl-4-piperidone hydrochloride 98% (TEMP) (from Aldrich). This probe, which has been shown to be specific for $^1O_2$ detection,[24–26] reacts with $^1O_2$ to yield a stable nitroxide radical 4-oxo-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (4-*O*-TEMPO), having a known 3-line EPR spectrum due to $I = 1$ of the $^{14}N_1$ atom.



(1)

TEMP (0.03 M, unless otherwise stated) or the stable radical 2,2,6,6-tetramethyl piperidine-*N*-oxyl (TEMPO) (from Sigma) (10^{-6} M) was added to

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10^7-10^9 cells/ml and then 100 μl from the sample were drawn by a syringe into a gas-permeable Teflon capillary (Zeus Industries, Raritan, NJ) with a 0.032 in. inner diameter and a 5.9 in. length. Each capillary was folded twice, inserted into a narrow quartz tube open at both ends (which ensures the presence of enough oxygen during illumination), and then placed into the EPR cavity, as previously described.[31,32]

To oxidize the hydroxylamine, which is produced upon reduction of 4-*O*-TEMPO, we added about 1–4 mM potassium ferricyanide (from Sigma) to post-illuminated cells.

To measure the effect of O_2 on the EPR signal during illumination, we bubbled O_2 through the quartz tube.

The EPR spectra were recorded on a Bruker ER 100d X-band spectrometer during or after illumination. The measurements were repeated at least four times for each sample. The microwave source of the EPR was set at 9.7 GHz and the power at 20 mW. Modulation frequency and modulation amplitude were 100 KHz and 1 G, respectively. The time constant was 655 ms, conversion time 82 ms and measurement time 168 s, unless otherwise stated. After acquisition, the spectra were processed by the Bruker WIN-EPR software version 2.11 for baseline correction, noise filtration, and integration of the peak signals. In all cases, the intensity was expressed in arbitrary units.

In order to calculate the significance of the percentage of increases in the triplet peak areas following irradiation, we used the paired *t*-test.

Smoothing the EPR Signal by Wavelet Transformation

In order to filter out non-EPR signal noises, we used the wavelet transformation procedure in the MATLAB environment (MATLAB wavelet toolbox). In this procedure, an orthogonal base function (ψ) [of a certain scale (s)] was chosen from a set of base functions with a qualitative similarity to the signal. In this case, the function "haar" was chosen because of its ability to smooth the raw signal (which is very noisy) and because it was the most suitable since it ascends and descends on a scale equal to half the width of the experimental Lorentian peak. This scale was determined from the mean of the 3 or 4 highest values obtained from the intervals between consecutive zero-crossings. Then the wavelet procedure was applied so that:

$$y(x) = \sum_s \int c(s,x)\psi\left(\frac{x-x'}{s}\right) \mathrm{d}x',$$

where $c(s,x)$ is the coefficient of the scale s at the position x.

The coefficients are then smoothed and exhibit peaks where the raw signal has one ascent and one descent on the scale s. The noise-free signal was calculated for the 3 (for Figs. 1–3, 5 and 7) or 4 (for Fig. 4) highest peak heights. Gaussian windows at these 3 or 4 peak locations, with width s and unit height, are then applied to the raw signal, preserving the shape of the raw signal at these points, and attenuating it elsewhere. The resulting noise-free spectra are presented here together with the original signal.

RESULTS

Light-induced Singlet Oxygen Formation Monitored by 4-*O*-TEMPO Detection

In spite of the difficulties discussed above, we succeeded in detecting an increase in the 4-*O*-TEMPO triplet ($a_N = 15.6$ G) spectra during illumination of NIH (Fig. 1) and in sperm cells (Fig. 2). Illumination of TEMP in cell-free PBS did not result in an increase of 4-*O*-TEMPO signal (Fig. 3). As the signal-to-noise ratio is low in all our measurements, we performed a wavelet transformation of the signal in a Matlab environment. All the spectra with subscript 1 are the reconstructed spectra after wavelet transformation; for example, in Fig. 1a and 1a1, the baseline and the reconstructed noise-free baseline signal in NIH are presented. The wavelet transformation procedure allowed us to eliminate the noise by reconstructing only 3 or 4 peaks, which have the highest similarity to a differential peak. Notice that as illumination of PBS solution in the presence of TEMP did not cause a 4-*O*-TEMPO signal (Fig. 3b), its noise-free spectra also did not give a symmetric triplet (Fig. 3b1). In all the figures, background noise arises mostly from the TEMP spin trap. Illumination of the NIH sample increased the 4-*O*-TEMPO signal, as shown in Fig. 1a,b, and their respective noise-free spectra Fig. 1a1,b1. The net increase in the spectra due to illumination is shown in Fig. 1c and its noise-free spectrum Fig. 1c1. We have calculated the average from several net spectra [(during/after illumination spectra) minus (before illumination spectra)]. The resulting average for illumination of NIH in the presence of TEMP is shown in Fig. 1d and its noise-free spectrum in Fig. 1d1. Illuminating NIH cells in the presence of sodium azide, an intracellular $^1O_2$ scavenger[33] (also used as a respiratory chain blocker[34]), results in a decrease in the 4-*O*-TEMPO signal, as can be seen in Fig. 1e and its noise-free spectrum Fig. 1e1. The subtracted spectrum is monitored in Fig. 1f and its noise-free spectrum in Fig. 1f1. Illumination of sperm cells also resulted in a small increase in the 4-*O*-TEMPO triplet signal. A small increase in the 4-*O*-TEMPO signal was noticed after 3 min of illumination (Fig. 2b) as well as in its noise-free spectra (Fig. 2b1), while 12 min of

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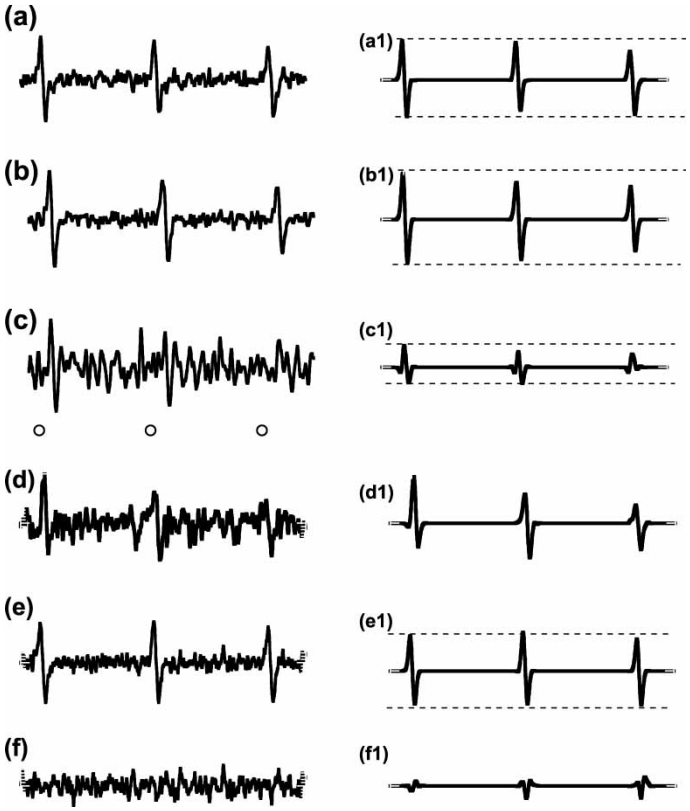

FIGURE 1 Detection of the increase in the 4-*O*-TEMPO signal in illuminated fibroblasts. (a) 10^6 NIH cells/ml + 80 mM TEMP without illumination; (b) with 4 min illumination; (c) subtraction spectrum (b) − (a); (a1) and (b1) are noise-free spectra obtained by using the wavelet transformation to (a) and (b), respectively; (c1) a subtracted spectra of (b1) − (a1); (d) average spectrum of the net signal [(after/during illumination NIH + TEMP) minus (before illumination of NIH + TEMP)] of six separate experiments (the o is assigned for the $a_N = 15.6$ G triplet); (e) 10^6 NIH cells/ml + 80 mM TEMP during illumination in the presence of 2 mM NaN_3 and its noise-free spectra (e1); (f) a subtraction spectrum (e) − (a); and (e1) the subtracted noise-free spectrum (e1) − (a1); percentage increase of the triplet peak area due to illumination was calculated to be 46 ± 13%, $p < 0.006$, for four experiments.

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illumination resulted in a larger increase in the 4-*O*-TEMPO signal (Fig. 2d,e). Together with the increase in the 4-*O*-TEMPO signal, an increase in the ascorbyl free radical (AFR) doublet ($a_H = 1.8$ G) signal was noticed during illumination (Fig. 2b,a1,b1). Therefore, the intensity of the middle peak was enhanced due to the overlap with the AFR doublet. The AFR is known to generate upon oxidation of ascorbate (a common nutrient found in cells) by 4-*O*-TEMPO to yield 1-hydroxyl-4-oxo-2,2,6,6-tetramethylpiperidine (4-*O*-TEMPO-H).[35,36]

$$\text{ascorbate} + \text{4-}O\text{-TEMPO} \rightarrow \text{4-}O\text{-TEMPO-H} + \text{AFR} \quad (2)$$

Depletion of the TEMPO Signal in Illuminated Cell Cultures

In the previous figures (Figs. 1 and 2), we succeeded in detecting an increase in the 4-*O*-TEMPO signal, which resulted from trapping the light-induced $^1O_2$ in various cells. However, we noted that this 4-*O*-TEMPO triplet signal decreases with time. For example, the intensity of the 4-*O*-TEMPO signal detected in NIH cells during illumination (Fig. 1) showed a small decrease, as a function of the illumination time, from the first low-field to the second high-field line (Fig. 1b,b1,c,c1). To illustrate the fact that illumination enhances the decay of the nitroxide signal, we introduced exogenous TEMPO, which is known to be reduced by intracellular agents,[35] and followed its decay with time. While there was no change in the TEMPO signal for more than 4 min in the cell culture (Fig. 4b), 83 s of illumination caused a significant decrease in the signal's intensity (Fig. 4c). In contrast, the TEMPO signal in the illuminated cell-free buffer was stable for a long time and was not influenced by illumination (Fig. 4d–f). Introducing DABCO, which reacts with $^1O_2$, did not prevent the depletion of the TEMPO signal in illuminated cells (data not shown). Adding of superoxide dismutase (SOD) inhibited, though not significantly, the decay of the TEMPO signal in illuminated sperm cells (probably by trapping $O_2^{\cdot-}$ produced in the membrane through sensitization of endogenous membrane photosensitizers located in the membrane NADPH oxidase complex) (data not shown). This may suggest that the TEMPO signal is not only depleted by light-induced

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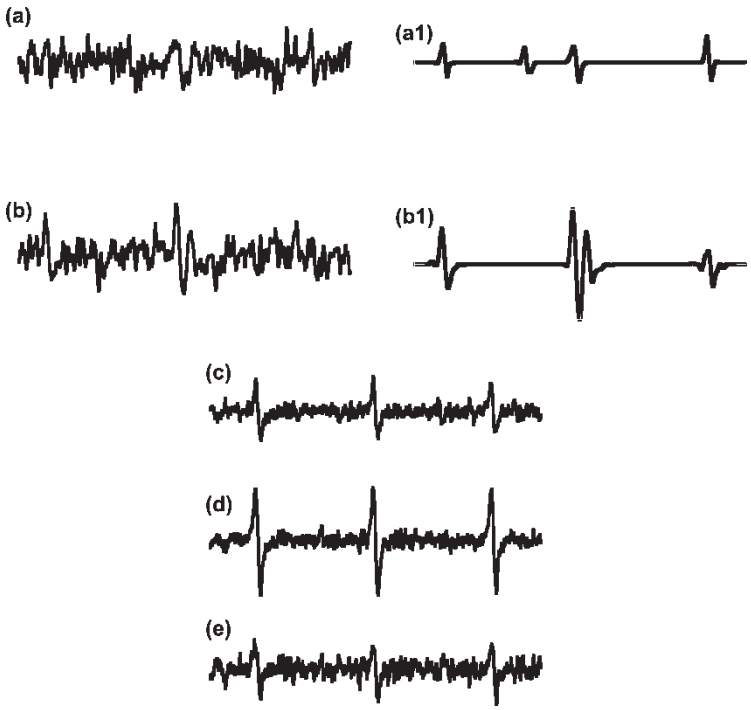

FIGURE 2 Detection of the increase in the 4-*O*-TEMPO signal in illuminated sperm cells. (a) 2.5×10^8 cells/ml + 23 mM TEMP without illumination; (b) with 3 min illumination; (a1) and (b1) are noise-free spectra obtained by using wavelet transformation to (a) and (b), respectively. Notice the overlap of the AFR doublet and the second peak of the 4-*O*-TEMPO signal; (c) 10^8 cells/ml + 60 mM TEMP without illumination; (d) after 12 min illumination; and (e) a subtracted spectra of (d) − (c); this spectrum was taken with TC = 1310 ms, conversion time = 328 ms and sweep time = 671 s. Percentage increase of the triplet peak area due to illumination was calculated to be 48 ± 25%, $p \leq 0.0.02$, for five experiments.

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radicals, which may reduce or oxidize the nitroxide, but also due to other reactions that enhance cellular reducing activity. It is known that the decay of a nitroxide signal in cellular systems results mainly from its bioreduction to the corresponding hydroxylamine (R_2NOH), which are EPR-silent.[35,37–40]

$$R_2NO^{\cdot} + H^+ + e^- \rightarrow R_2NOH \qquad (3)$$

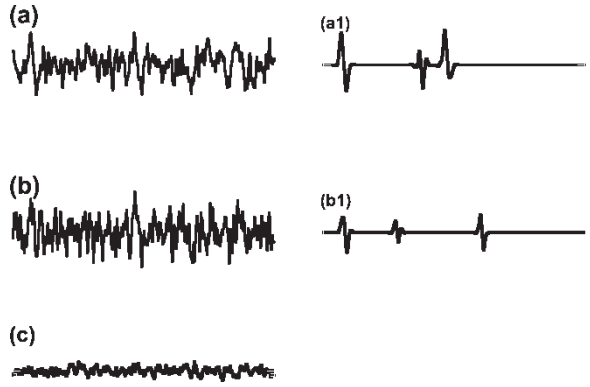

FIGURE 3 Detection of the increase in the 4-*O*-TEMPO signal in cell-free PBS. (a) PBS + 23 mM TEMP without illumination; (b) with 3 min illumination; (a1) and (b1) are noise-free spectra obtained by using the wavelet transformation to (a) and (b), respectively; and (c) average spectrum of the net signal [(after/during illumination of PBS + TEMP) minus (before illumination of PBS + TEMP)], from seven separate experiments.

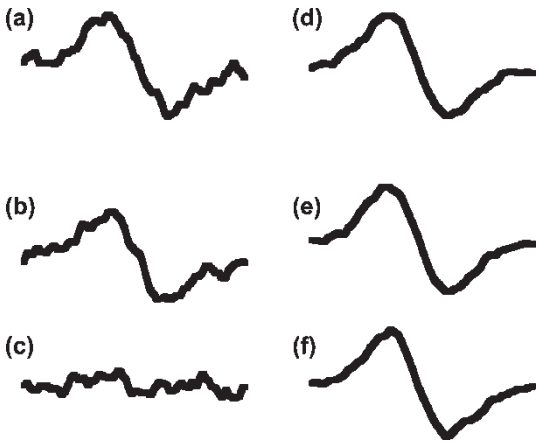

FIGURE 4 Illumination causes quenching of 10^{-6} M TEMPO exogenously added to sperm cells. TEMPO + sperm cells (a–c): (a) without irradiation; (b) after 270 s (without irradiation); (c) during 83 s of irradiation; TEMPO + PBS (d–f): (d) without irradiation; (e) after 270 s (without irradiation); and (f) during 83 s of irradiation. Note that only the first low field peak is monitored. These data were taken with receiver gain = 2.8×10^4, TC = 2621 ms, conversion time = 164 ms, and sweep time = 83 s.

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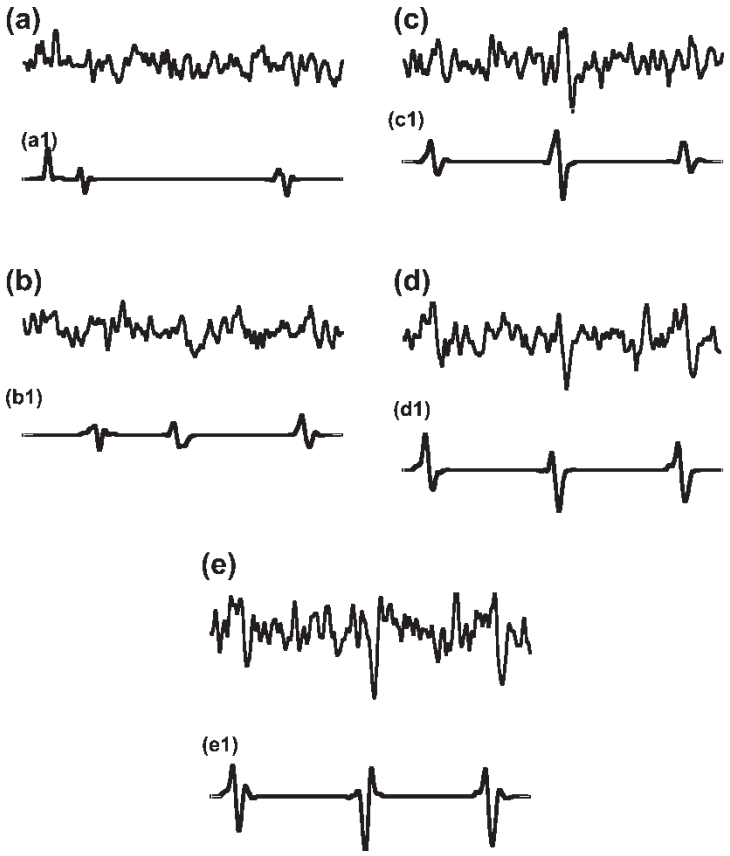

FIGURE 5 Quenching the 4-*O*-TEMPO signal in illuminated fibroblasts and recovery of the signal by ferricyanide. 10^6 NIH cells/ml + 70 mM TEMP: (a) without illumination (b) with 6 min illumination; (c) and (d) are measured after addition of 4 mM ferricyanide to (a) and (b), respectively; (e) subtracted spectrum (d) − (c); (c1) and (d1) are noise-free spectra obtained by using the wavelet transformation to (c) and (d), respectively; and (e1) subtracted spectra of (d1) − (c1). [Note the increase in the intensity of the middle peak in (c) and (c1) due to oxidation of endogenous ascorbate by ferricyanide. This increase in the intensity of the middle peak of (c) also causes a negative subtracted peak in the middle peak of (e) and (e1)].

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Detection of Light-induced $^1O_2$ by Recovery of the 4-*O*-TEMPO by Ferricyanide

As low-energy visible light (LEVL) (400–800 nm) facilitates the reduction of 4-*O*-TEMPO to 4-*O*-TEMPO-H, we used the one-electron weak oxidant $K_3Fe(CN)_6$ to oxidize the hydroxylamine back to 4-*O*-TEMPO in illuminated fibroblasts and sperm cells. The addition of ferricyanide to the fibroblasts, which have been illuminated in the presence of TEMP, recovered the 4-*O*-TEMPO signal (Fig. 5d and its noise-free spectrum Fig. 5d1). This recovery exceeded the recovered 4-*O*-TEMPO signal in non-illuminated cells (Fig. 5c and its noise-free spectrum Fig. 5c1). The net recovered signal after subtracting the recovered non-illuminated cell baseline (spectra 5d minus spectra 5c) is presented in Fig. 5e showing the EPR triplet of the nitroxide and its subtracted noise-free spectrum (Fig. 5e1). To improve the spectral resolution of 4-*O*-TEMPO detected upon illumination of sperm cells, the measurement range was limited to the low-field line alone (Fig. 6). Figure 6a,b shows that illumination caused a decrease in the double integral of the nitroxide baseline signal from a value of 0.9 to 0.55 (paired *t*-test ≤ 0.02). Addition of ferricyanide increased the last value to 1.76 (Fig. 6d), as compared to the addition of ferricyanide to a non-illuminated sample which resulted in recovery of the background signal to a value of 1.19 only (Fig. 6c) (paired *t*-test ≤ 0.55). Subtracting the recovered background signal (Fig. 6c) from the recovered signal of the illuminated samples (Fig. 6d), reflects the net increase of the 4-*O*-TEMPO after illumination (Fig. 6f.).

Increase in the 4-*O*-TEMPO Signal in Illuminated cells under Oxygen

Keeping a constant flow of oxygen during illumination (up to 10 min) of fibroblasts increases the 3-line signal of 4-*O*-TEMPO (Fig. 7d–h). Illuminating TEMP in cell-free PBS with or without oxygen flushing did not enhance signal intensity (Fig. 7b,c). Although the reduction of 4-*O*-TEMPO during illumination of the cells prevents its accumulation (as was noticed in our measurements (Figs. 4–6)), its signal intensity increased progressively in oxygen-saturated medium. The presence of oxygen during

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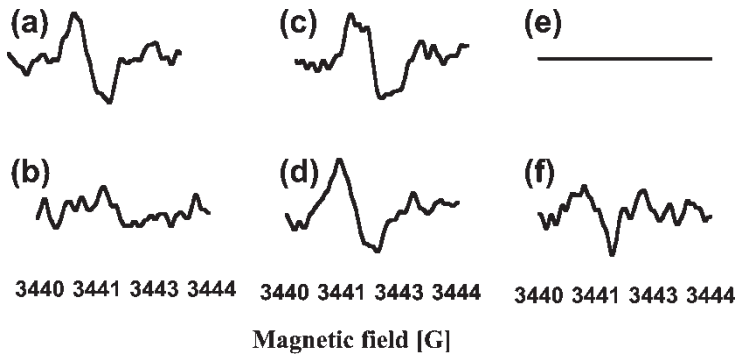

FIGURE 6 Quenching the 3441 G peak of the 4-*O*-TEMPO signal in 10^{10} sperm cells/ml during 3 min illumination and recovery of the signal by 1 mM ferricyanide. Sperm cells + 20 mM TEMP: (a) no illumination; (b) with illumination; (c) and (d) after addition of ferricyanide to (a) and (b), respectively; (e) and (f) after subtracting the recovered base line signal (c) from (c) and (d), respectively; The peak integration area was calculated from four separate similar experiments in a.u.: (a) 0.94 ± 0.29, (b) 0.55 ± 0.21, (c) 1.19 ± 0.13, and (d) 1.76 ± 0.43 [paired *t*-test for (a) compared to (b) $t \leq 0.002$ and for (c) compared to (d) $t \leq 0.55$]. These data were taken with time constant = 1311 ms.

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illumination of the cells increased the production rate of singlet oxygen, and consequently, that of the 4-*O*-TEMPO signal in these cells. On the other hand, the reduction rate was not increased to the same extent. Moreover, oxidation of the corresponding hydroxylamine back to 4-*O*-TEMPO occurs constantly by O_2, thus an increase in the 3-lines signal was obtained.

Increase in the 4-*O*-TEMPO Signal in Illuminated Cells Enriched with Hematoporphyrin

A clear signal of 4-*O*-TEMPO (Fig. 8) was obtained in illuminated fibroblasts which had been enriched with hematoporphyrin, a photosensitizer which is known to generate $^1O_2$ upon illumination.[41] (Note that the *y*-scale in Fig. 8 differs from that of Fig. 1).

DISCUSSION

One of the ways to explain the biostimulative effects of LEVL is by assuming that small amounts of singlet oxygen and other reactive oxygen species (ROS) are produced during illumination of the cell.[12–14,42,43] In the present study, we have succeeded in showing that LEVL can indeed produce $^1O_2$ in the living cell.

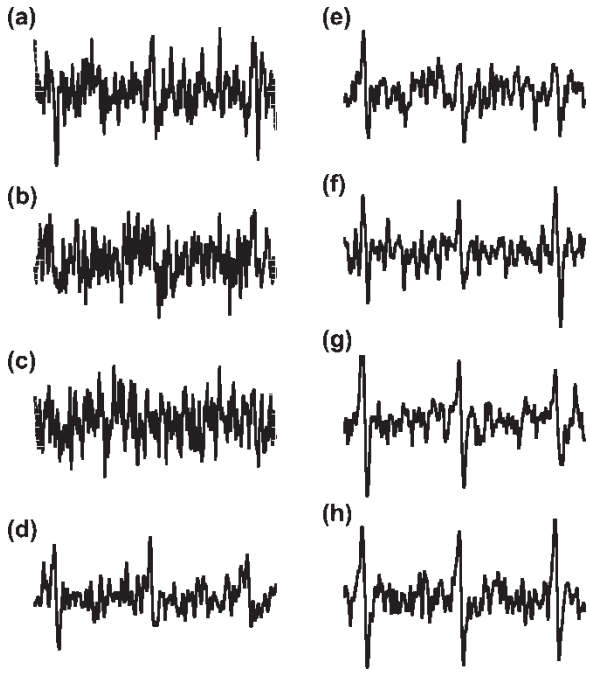

FIGURE 7 Increase in the 4-*O*-TEMPO signal as a function of illumination time in fibroblasts saturated with oxygen. PBS + 23 mM TEMP: (a) before illumination; (b) during 3 min illumination; (c) during 5.5 min illumination in the presence of oxygen bubbling; 10^7 NIH cells/ml + TEMP (23 mM) with illumination of: (d) 0.5, (e) 3, (f) 5.5, (g) 8 and (h) 10 min.

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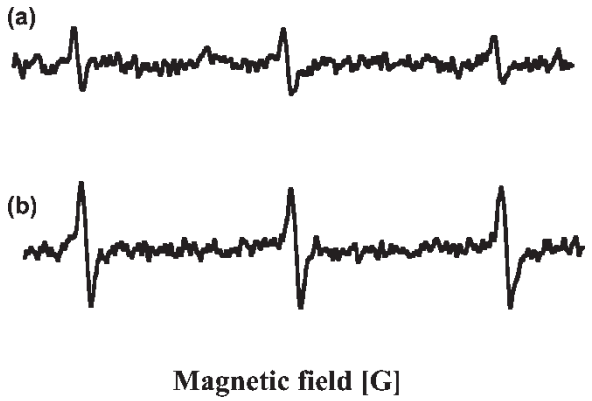

FIGURE 8 Detection of the increase in the 4-*O*-TEMPO signal in illuminated fibroblasts enriched with hematoporphyrin. (a) 10^6 NIH cells/ml loaded with hematoporphyrin + 80 mM TEMP without illumination; (b) with 4 min illumination.

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In order to interact with the living cell, light must be absorbed by intracellular chromophores. Endogenous porphyrins, such as mitochondrial cytochromes, pyridine cofactors, NADPH/NADH and flavoproteins, have been previously proposed as the chromophores responsible for photobiostimulation[12,13,44,45] since they possess absorption bands in the visible range. These chromophores can serve as photosensitizers that generate ROS following illumination.[5,45–47] Singlet oxygen is produced by a type II reaction where the energy needed to excite molecular oxygen from its triplet ground state to the first singlet state is provided by the excited triplet state of the photosensitizer.[6] Although high ROS flux, which can be obtained by enriching the cells with exogenous photosensitizers, impairs cells (by lipid peroxidation and ATP depletion) and thus is used in photodynamic therapy (PDT) for killing tumor cells, recent evidence has demonstrated that lower ROS fluxes play an important role in the activation and control of many cellular processes, such as transcription factor release, gene expression, muscle contraction, and cell growth.[48–52] Thus, ROS should not be merely perceived as agents that damage cells, but perhaps also as mediators of physiological functions and as secondary messengers.

Only a few studies demonstrating ROS formation in cell cultures by LEVL without exogenous photosensitizer addition have been reported. For example, Callaghan, *et al.*[42] found that light at 660 nm can induce the formation of $O_2^{\cdot -}$ and H_2O_2 in a hemopoietic cell line. Hydrogen peroxide was also detected spectrophotometrically in illuminated sperm cells,[53] and recently we have detected $^{\cdot}OH$ in cells illuminated with visible light.[54,55]

As the steady state concentration of $^1O_2$ in cells is low, there is only vague evidence in the literature for its formation in illuminated cell cultures. Nevertheless, in the present work, we have detected a small signal of 4-*O*-TEMPO, which reflects $^1O_2$ formation in illuminated sperm cells and fibroblasts (Figs. 1 and 2). Comparing the signals of illuminated NIH cells with those of illuminated cells enriched with hematoporphyrin shows that indeed a larger signal is obtained in the latter (Fig. 8). Whereas LEVL illumination does not cause any cell damage, illumination of hematoporphyrin-enriched cells damages the cell through $^1O_2$ production.[56]

Our success in detecting 4-*O*-TEMPO in illuminated cells is partially due to the use of a gas-permeable Teflon capillary tube as the sample compartment. The advantages of the Teflon capillary are: (a) it contains a small quantity of water (water has a high dielectric constant, which lowers the sensitivity of detection by the EPR) and (b) it ensures the presence of enough oxygen in the examined sample.

The present detection of $^1O_2$ formation in illuminated cells is important since $^1O_2$ has pronounced effects on cellular signaling.[5,6,57] A recent paper entitled "Harmful $^1O_2$ can be helpful" provides *in-vitro* documentation on the ability of $^1O_2$ to modify cellular behavior.[58]

Together with $^1O_2$ production, we found that LEVL also increases the reducing ability of the cells, as can be seen from the light-induced rapid depletion of the stable radical TEMPO (Fig. 4). This rapid reduction of 4-*O*-TEMPO to 4-*O*-TEMPO-H upon its production is also a reason for its limited detection in illuminated cells. Addition of ferricyanide, which is a mild oxidant, into cells where illumination in the presence of TEMP resulted in the quenching of the 4-*O*-TEMPO triplet caused a partial chemical recovery of the 4-*O*-TEMPO signal (Figs. 5 and 6). The reduction of the 4-*O*-TEMPO signal was also observed in some of the signals obtained from the illumination of hematoporphyrin-enriched fibroblasts in the presence of TEMP (data not shown).

There are several potential sources for the reduction of 4-*O*-TEMPO or TEMPO in illuminated cells. (a) The electron transport chain of the mitochondria, especially at the ubiquinol site.[59–61] It is known that LEVL accelerates the mitochondrial electron chain,[8] thus increasing the reduction of 4-*O*-TEMPO to 4-*O*-TEMPO-H. (b) Light-induced production of $O_2^{\cdot -}$;[45] the protonated[53] radical, $HO_2^{\cdot}$ can oxidize the 4-*O*-TEMPO to the oxo-ammonium cation which may react with species such NADH or NADPH to form the corresponding hydroxylamine.[62] (c) Excited riboflavin/flavin photosensitizers can reduce the nitroxide, thus producing significant amounts of hydroxylamines.[63] Therefore, flavins can also participate in the increase of 4-*O*-TEMPO reduction by LEVL.

Since illumination causes two opposing effects, the production of 4-*O*-TEMPO (Figs. 1 and 2) and its reduction to an EPR-silent 4-*O*-TEMPO-H signal (Figs. 5 and 6), we flushed O_2 during illumination in

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order to increase the $^1O_2$ steady state concentration. It can be seen that the production of 4-*O*-TEMPO in the presence of oxygen was increased as a function of illumination time (Fig. 7). This result indicates that $^1O_2$ can be produced in response to visible light via a type II photochemical reaction. Nevertheless, singlet oxygen may also be produced from $O_2^{\cdot-}$ (also found to be generated in illuminated cell cultures[40] by a charge transfer reaction, in the presence of metals):

$$H_2O_2 + O_2^{\cdot-} \rightarrow OH + OH^- + {}^1O_2 \text{[64]} \qquad (4)$$

Mammalian cells exist under a constant oxidative siege, requiring an appropriate balance of oxidants and antioxidants. Induction of either can modulate biological processes.[48,53,65–67] The present findings show that visible light stimulates production of oxidants, such as singlet oxygen, and at the same time increases the reductive capability of the cells. The change in the redox state of the cell may explain the photobiostimulative effects.

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Acknowledgements

We would like to thank Dr Lev Weiner from the Weizmann Institute of Science, Israel, for his assistance in the spin trapping EPR technique.

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