Low Energy Visible Light Induces Reactive Oxygen Species Generation and Stimulates an Increase of Intracellular Calcium Concentration in Cardiac Cells

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Summary

Low energy visible light (LEVL) irradiation has been shown to exert some beneficial effects on various cell cultures. For example, it increases the fertilizing capability of sperm cells, promotes cell proliferation, induces sprouting of neurons and more. In order to learn about the mechanism of photobiostimulation, we studied the relationship between increased intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) and reactive oxygen species (ROS) production following LEVL illumination of cardiomyocytes. We found that visible light causes the production of O\textsubscript{2}^{-} and H\textsubscript{2}O\textsubscript{2}, and that exogenously added H\textsubscript{2}O\textsubscript{2} (12 µM) can mimic the effect of LEVL (3.6 J/cm\textsuperscript{2}) to induce a slow and transient increase in [Ca\textsuperscript{2+}]\textsubscript{i}. This [Ca\textsuperscript{2+}]\textsubscript{i} elevation can be reduced by verapamil, a voltage dependent calcium channel inhibitor. The kinetics of [Ca\textsuperscript{2+}]\textsubscript{i} elevation and morphologic damage following light or addition of H\textsubscript{2}O\textsubscript{2} were found to be dose dependent. For example, LEVL, 3.6J/cm\textsuperscript{2}, which induced a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} did not cause any cell damage, while visible light at 12J/cm\textsuperscript{2} induced a linear increase in [Ca\textsuperscript{2+}]\textsubscript{i} and damaged the cells. The linear increase in [Ca\textsuperscript{2+}]\textsubscript{i} resulting from high energy doses of light could be attenuated into a non-linear small rise in [Ca\textsuperscript{2+}]\textsubscript{i} by the presence of extracellular catalase during illumination. We suggest that the different kinetics of [Ca\textsuperscript{2+}]\textsubscript{i} elevation following various light irradiation or H\textsubscript{2}O\textsubscript{2} treatment, represents correspondingly different adaptation levels to oxidative stress. The adaptive response of the cells to LEVL represented by the transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} can explain LEVL beneficial effects.
Introduction

Life on earth is entirely dependent upon the interaction of sunlight with cells especially in plant photosynthesis (1). Sunlight also has medical benefits, which have been exploited for over thousands of years in ancient Egypt, India and China in treating skin diseases, psoriasis, vitiligo and even cancer (2). Recent observations show that even low energy visible light (LEVL) can serve as a medical tool. For example, LEVL increases the rate of wound healing (3), enhances the fertilizing capability of sperm cells (4) and increases the rate of healing bone defects (5). *In vitro* studies have found that LEVL increases proliferation of cells as fibroblasts (6), keratinocytes (7), and lymphocytes (8), and induces the respiratory burst in neutrophils (9). The mechanism of photobiostimulation by LEVL is still unclear. It has been suggested that reactive oxygen species (ROS), which can be produced by photosensitization of endogenous cell chromophores such as cytochromes (10), flavins/riboflavins (11) and NADPH (12), may have an important role in this light/tissue interaction (13-15). The suggestion is based on the recent recognition that small amounts of ROS are considered to be important for mediating cell activities (16-19). The production of ROS in response to low energy visible light has been demonstrated in fibroblasts (20), sperms (21) and lymphocytes (15). In addition, it has been found that LEVL causes \([Ca^{2+}]_i\) elevation in cells like sperm (4) and skin (22). Transient increases in \([Ca^{2+}]_i\) initiate cellular signaling pathways such as activation of photoreceptors and induction of growth factors and contractility (23, 24). Thus a change in \([Ca^{2+}]_i\) following LEVL may be another important mediator of photobiostimulation effects.

The linkage between \([Ca^{2+}]_i\) and the redox state of the cell is well known in controlling many cellular systems. For example, it has been found that H\(_2\)O\(_2\) when added to skeletal muscle fibers causes an increase in \([Ca^{2+}]_i\), which is prevented by a reducing agent (25). Incubation of endothelial cells with H\(_2\)O\(_2\) caused an increase in \([Ca^{2+}]_i\), followed by myosin phosphorylation and cell contractions (26). Growth
factors and hormones were shown to stimulate ROS production, which were dependent on $[\text{Ca}^{2+}]_i$ rise (27). The relationship between ROS and $[\text{Ca}^{2+}]_i$ has been suggested to involve the redox-sensitive transcription factor N kappa $\beta$, which was found to change $[\text{Ca}^{2+}]_i$ homeostasis in response to changes in the redox state of thiol groups (28). The kinetics which characterizes the $[\text{Ca}^{2+}]_i$ elevation has been shown to be an important parameter determining the kind of signal which will be evoked. Livingston et al (29) showed that high concentrations of oxidants (>50 $\mu$M) caused a sustained increase in $[\text{Ca}^{2+}]_i$, whereas a transient increase in $[\text{Ca}^{2+}]_i$ was observed following administration of a low concentration of oxidants. More than a fourfold increase in the $[\text{Ca}^{2+}]_i$ level was obtained in photodynamic (PD) treatment of mouse myeloma cells that had been enriched with exogenous photosensitizers before illumination, whereas only a slight increase in $[\text{Ca}^{2+}]_i$ was observed in irradiated cells without exogenous photosensitizers (30).

Only few works have dealt with Ca/ROS changes following LEVL irradiation. In this study, we have investigated the relationship between low energy light, ROS and $[\text{Ca}^{2+}]_i$ levels in cardiac cells. We chose cardiomyocytes, as low energy photoirradiation has been found to improve heart preservation for transplantation (31). In addition, cardiomyocytes possess low levels of antioxidant enzymes thus being more sensitive to light and ROS.

Materials and Methods

Chemicals

Indo-1/AM, (purchased from Molecular Probes Inc., USA), 5-(diethoxyphosphoryl)-5-methyl–1-pyrroline-N-oxide (DEPMPO), (purchased from Megapharm, Alexis USA), 2,2,6,6-tetramethyl-4-piperidone (TEMP), (obtained from Aldrich). All other chemicals were purchased from Sigma.
Fluorescence measurements of rat cardiac cells

Rat cardiac cells, (1-2 day-old) were grown for 3-6 days on a cover glass coated with gelatin/collagen, as previously described (32). The cultured cells were then incubated in the dark for 30-50 min with 2 µM of the fluorescence probe indo-1/AM and 1.5 µM pluronic acid in glucose-enriched phosphate-buffered saline (PBS) at room temperature. The cardiac cells were then washed with glucose-enriched PBS and transferred to a chamber over a Zeiss inverted microscope (Axiovert 135 TV). The microscope was focused on a single cardiomyocyte or a group of two to three cells in the indo-1 loaded culture. Every 2-5 minutes the average fluorescence was measured for 10 seconds by using software written by D. Kaplan, Biological Institute Ness Ziona, Israel, as previously described (33).

Illumination and H2O2 treatment

While on the chamber of the microscope, the cells were irradiated from 1-5 minutes with a filtered homemade light source (400-800 nm), at 40 mW/cm². Alternatively, cells were treated with 12-48 µM H2O2 by replacing the medium with glucose-enriched PBS containing H2O2.

Involvement of L-type calcium channel

To determine whether changes in intracellular calcium are mediated by a specific calcium-channel, 10 µM of verapamil (L-type voltage-dependent calcium channel blocker) were added to cardiac cells before LEVL irradiation or H2O2 treatment and then the indo-1 fluorescence was measured.

ROS measurements

Measurements of hydrogen peroxide by luminol. Cell suspension of 200 µL (containing 10⁶ cells) were added to a 96-multi-well cluster dishes. Each well was then illuminated and immediately, horseradish peroxidase (HRP) (2.4 unit/ml) and luminol (20 µM) were added. The luminescence of each well was measured by a
TECAN spectrofluorimeter at room temperature every 30-60 seconds during a period of 5 min. The peak value of the luminescence as a function of time was taken after subtracting the value of the blank signal (luminescence of the illuminated medium).

**Measurements of superoxide anion radicals by Electron Paramagnetic Resonance (EPR)-spin trapping technique.** To measure $O_2^-$ we used the EPR-spin trapping technique with the spin trap DEPMPO (34). The DEPMPO, reacts with hydroxyl radicals to produce DEPMPO-OH or with superoxide anion radicals to produce the spin adduct DEPMPO-OOH (35). The latter is a relatively stable paramagnetic species (half life time, 17.7 min; (36)) having two conformers with a characteristic EPR spectrum of 18 lines. To simplify the spectrum and to increase its resolution, only the parts of the spectrum where DEPMPO-OOH lines are distinguishable from DEPMPO-OH lines were scanned.

Samples of $1.2 \times 10^6$ cells/ml with DEPMPO (0.02 M) were drawn by a syringe into a gas-permeable teflon capillary (Zeus, Raritan, NJ) and inserted into a narrow quartz tube which was open at both ends (37). Then the tube was placed into the EPR cavity and the spectra were recorded on a Bruker EPR 100d X-band spectrometer while illuminating the samples in the EPR cavity. The EPR measurement conditions were as followed: frequency: 9.75 GHz; power: 20 mW; scan width: 25 G; resolution: 512; receiver gain: $2 \times 10^5$; conversion time: 164 ms; time constant: 2622 ms; # of scans: 4; illumination time: 83 seconds.

**Using EPR-spin trapping technique to measure hydroxyl radicals and singlet oxygen production by sensitization of Indo-1/AM.** Although, the fluorescence probe Indo-1 was used to measured $[Ca^{2+}]_i$, as its excitation length (355 nm) is not overlap with the wavelength rang of our light source treatment. To excluded any possibility that Indo-1 can be a photosensitize by the treated light source, we measured the ESR-spin trap spectrum of Indo-1/AM while it was illuminated. Neither production of $^1O_2$ nor did production of $O_2^-$ or $OH$ was obtained by using the spin traps TEMP and
5,5-dimethyl-1-pyrroline-N-oxide (DMPO) respectively (these data will not be presented).

**Structural changes following illumination or H$_2$O$_2$ treatment**

Myocytes in PBS were treated with increasing concentrations of hydrogen peroxide or were illuminated with different doses of light. After 50 min, the cells were returned to growth medium and placed in a 5% CO$_2$ incubator environment at 37°C. The cells were fixed after 24 hr, and then immunohistochemical staining was performed using mouse monoclonal anti-α-sarcomeric actin(C-5) and goat anti-mouse biotinylated immunoglobulin conjugated with extrAvidin peroxidase (immunohistochemical kit, IMMH-1). Following staining with chromogen 3-Amino-9-ethylcarbazole (AEC), the cells were counterstained with hematoxylin (32).

**Lactate dehydrogenese (LDH) assay**

Cytotoxicity was assessed by activity of released LDH into the culture medium. The LDH activity was measured using an LDH kit as described before (38). The results are expressed as percent of LDH released in samples relative to samples in which cells were lysed with 1% Triton x-100.

**Results**

**ROS production by cardiomyocytes**

In order to verify the generation of ROS in response to visible light we measured H$_2$O$_2$ via luminol, and O$_2^{•−}$ by the EPR spin trapping technique using DEPMPO. Broad band visible light illumination (3.6 J/cm$^2$) of cardiomyocyte suspension in the presence of luminol, resulted in a significant increase of 75±20% (p$\leq$ 0.047) in the luminescence of luminol (Fig. 1). This indicates that LEVL illumination increases the H$_2$O$_2$ concentration in the cells. The EPR technique confirms the presence of O$_2^{•−}$. In Figure 2a a simulated spectrum of DEPMPO after trapping O$_2^{•−}$ to form DEPMPO-
OOH is shown in the detectable range. Illuminating cardiomyocytes while scanning the EPR spectrum for 83 seconds, resulted in the appearance of a spectrum which is compatible with that of DEPMPO-OOH (Fig. 2b), while the non-illuminated cell suspension spectrum exhibited only background noise (Fig. 2c). Addition of superoxide dismutase (SOD), a superoxide oxide anion scavenger, to the cell suspension decreased the intensity of the DEPMPO-OOH spin adduct signal (Fig. 2d). These results show that LEVL illumination increases the level of $O_2^{-}$ in illuminated cardiomyocytes.

**Effect of visible light irradiation on $[Ca^{2+}]_i$**

We next determined whether light could directly induce increased intracellular $[Ca^{2+}]_i$ even without exogenous photosensitizers. We found that illumination at an energy density of 3.6 J/cm² cause a semi-transient elevation of $[Ca^{2+}]_i$, with a broad peak of 12% which lasted for more than 30 min followed by a decrease to a stable plateau of 8% above the control (Figs. 3b, 7a). Increasing the light energy density to 12 J/cm² resulted in a linear elevation of $[Ca^{2+}]_i$, which reached 25%, 60 minutes after illumination (Fig. 3c). Nevertheless, this elevation was reduced to 7 % above control, as a result of including 200 U/ml catalase (H$_2$O$_2$ scavenger) into the medium before illumination, (Fig. 3d). In the control, the observed $[Ca^{2+}]_i$ values fluctuated up to 3 % above and 2 % below the basal level during 80 minutes (Fig. 3a).

**Effect of H$_2$O$_2$ on $[Ca^{2+}]_i$**

To support our hypothesis that ROS take part in the pathway leading to the increase in $[Ca^{2+}]_i$, following visible light illumination, we next compared the effect of light with that of H$_2$O$_2$ on $[Ca^{2+}]_i$ changes. The kinetics of $[Ca^{2+}]_i$ changes after H$_2$O$_2$ treatment were found to be concentration-dependent and ranged from a transient increase in $[Ca^{2+}]_i$ to an exponential increase over time (Fig. 4). Although H$_2$O$_2$ at all concentrations was present in the medium during the measurement, 12-18 µM H$_2$O$_2$ caused a transient increase in $[Ca^{2+}]_i$, with a peak of 12% above the control, about
35 minutes after addition of H$_2$O$_2$ (Figs. 4a(small window), 8a). Addition of 48 µM H$_2$O$_2$ caused a moderate increase in [Ca$^{2+}$]$_i$, during the first 20 minutes, which was followed by a linear increase in [Ca$^{2+}$]$_i$ reaching a plateau of 150%, 50 minutes after its addition (Fig. 4c). A concentration of 24 µM H$_2$O$_2$ showed an intermediate behavior, the calcium oscillations were increased, reaching a maximum of 60%, 38 minutes after the addition of H$_2$O$_2$, the [Ca$^{2+}$]$_i$ didn’t return to the basal level but decreased to a semi-plateau 50% above the control (Fig. 4b). Comparing these results with those obtained with LEVL (Figs. 3b, 7a), indicates that 12µM H$_2$O$_2$ can mimic the effect of 3.6 J/cm$^2$ visible light and both cause a slow and a long lasting increase of [Ca$^{2+}$]$_i$. In both treatments, the kinetics of the [Ca$^{2+}$]$_i$ changes are dependent on the concentration of H$_2$O$_2$ or on the light energy doses.

**Effect of light or H$_2$O$_2$ on the viability of the cells**

To rule out the possibility that visible light, at the doses employed, causes damage to the cell membrane, we measured LDH secretion to the medium immediately, 2.5, 6 and 24 hours after 3.6 or 12 J/cm$^2$ visible light illumination (LDH is a biochemical marker for the integrity of the membrane (39)). We found that the amount of LDH released to the medium at various times after 3.6 J/cm$^2$ or 12 J/cm$^2$ illumination was similar to that of the LDH released in non-illuminated cultures (Fig. 5) and the increase in LDH level during 24 hours is only due to natural exocytotic release.

Another approach for determining the effect of light or H$_2$O$_2$, was to observe the cell morphology 24 hours after illumination or treatment with H$_2$O$_2$ (Fig. 6). The cultured cells were immunocytochemically stained for α-sarcomeric actin to observe the contractile filaments and were counterstained with hematoxylin to observe the nucleus. In the control (Fig. 6A), most of the cardiomyocytes were flattened with strands of well-organized myofibrils α-sarcomeric actin with evident cross-striation. The nucleus showed a well stained chromatin structure. Treatment with 12 µM of H$_2$O$_2$ maintained cross-striations but caused a slight disorganization of the myofibril
structure and a slight loss of $\alpha$-sarcomeric actin staining. No changes were shown in the nucleus (Fig. 6B). Increasing the $\text{H}_2\text{O}_2$ concentration to 24 $\mu$M caused focal disorganization of myofibrill structures, vacuolization of the cytoplasm (blue arrow) and picnotic damage to many nuclei (white arrow). Nevertheless, approx. 65% of cells did not exhibit changes (Fig. 6C). By further increasing the $\text{H}_2\text{O}_2$ concentration to 48 $\mu$M, a severe alteration of the $\alpha$-sarcomeric actin positive structure, disorganization of the myofibrils, vacuolization of the cytoplasm and perinuclear edema (blue arrow) was induced. The nucleus showed picnotic damage (white arrow) (Fig. 6D).

Illumination at 3.6 J/cm$^2$ caused no visible alteration in cardiomyocyte structure, as seen in stained $\alpha$-sarcomeric actin myofibrils and maintenance of cross-striation (Fig. 6E). Increasing the illuminating energy to 7.2 J/cm$^2$ caused a decrease in the $\alpha$-sarcomeric actin staining, but the nucleus was without visible changes (Fig. 6F). Increasing the illumination energy to 12 J/cm$^2$ caused a disorganization of the myofibril structures, decrease in $\alpha$-sarcomeric actin staining, vacuolization of the cytoplasm and oncotic damage (swelling) of the cytoplasm, though no visible damage to the nuclei was seen (Fig 6G). Adding exogenous catalase (200 U/ml) to the medium of cardiomyocytes culture before irradiation with 12 J/cm$^2$ protected the cells, as seen in Figure 5H.

We conclude that the toxic effect of visible light or $\text{H}_2\text{O}_2$ to cardiomyocytes is dose dependent. Illumination below 3.6 J/cm$^2$ or concentrations below 24 $\mu$M of $\text{H}_2\text{O}_2$ do not cause any damage to the majority of the cells 24 hours after treatment. Therefore, visible light illumination with energy less than 3.6 J/cm$^2$ appears safe for cardiomyocytes. Moreover, the kinetics of $[\text{Ca}^{2+}]_i$ measured with increasing doses of light or $\text{H}_2\text{O}_2$ treatment shows a good correlation with the viability of the cells. Mild treatment with light (3.6 J/cm$^2$) or a low $\text{H}_2\text{O}_2$ concentration (12 $\mu$M), which do not damage the cell (Fig. 6 E and B), cause a transient increase in $[\text{Ca}^{2+}]_i$ (Figs. 3b and 7a, Figs. 4a and 8a). Increasing the $\text{H}_2\text{O}_2$ concentration to 24 $\mu$M caused a damage to some of the cells (Fig. 6C) and changed the kinetics of $[\text{Ca}^{2+}]_i$ to oscillation, reaching
a plateau (Fig 4b). High doses of light 12 J/cm² or H₂O₂ 48 µM, which show a clear toxic effect on the cells 24 hours after treatment, (Fig. 6 G and D), were correlated with a linear increase in [Ca²⁺], (Fig 3c) or a semi-exponential increase, (Fig 4c) receptively. Furthermore, the presence of catalase, which reduces the H₂O₂ concentration, formed by visible light illumination, causes a change in the [Ca²⁺], kinetics. The linear kinetics observed after 12 J/cm² illumination changes into smaller oscillations in the presence of catalase (Fig. 3d), which also prevented cell damage (Fig. 6H). These results suggest that the kinetics of [Ca²⁺], can reflect the ability of the cells to adapt to changes due to H₂O₂ and light. Whereas, light treatment of 3.6 J/cm² and low H₂O₂ concentrations (12 µM) are doses which the cell tolerates.

**Effects of verapamil on [Ca²⁺], increase following LEVL or H₂O₂**

We have previously observed that the source for [Ca²⁺], elevation following LEVL illumination in sperm cells is Ca²⁺ influx from the external medium (4). To learn about the mechanism of [Ca²⁺], elevation following LEVL illumination in cardiomyocytes, we measured the [Ca²⁺], changes in cultured treated cells to which verapamil was added 15 min before treatment. Our results show that verapamil, which has no effect on [Ca²⁺], (Fig. 7c), can attenuate the increase in [Ca²⁺], after 3.6 J/cm² illumination (Fig. 7b) or 18 µM H₂O₂ addition (Fig. 8b). These results suggest that one of the ways by which low doses of visible light as well as low concentrations of H₂O₂ increase [Ca²⁺], in cardiomyocytes is by calcium influx through voltage dependent calcium channels. Nevertheless, as verapamil did not prevent completely the increase in [Ca²⁺], other possibilities are not excluded.
Discussion

In the present study the change in \([\text{Ca}^{2+}]_i\) in LEVL illuminated cardiomyocytes, its linkage to ROS production and to the cell viability is demonstrated. The kinetics of the increase of \([\text{Ca}^{2+}]_i\) has been found to reflect an adaptive response of the cells to oxidative stress.

Generation of ROS in LEVL illuminated cardiomyocytes

In (Figs. 1, 2) \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) formation in LEVL illuminated cardiomyocytes is shown, it is believed that ROS are produced through activation of endogenous photosensitizers. Therefore, the resulting ROS are spread all over the cell, in contrast to photodynamic therapy (PDT) treatment, where the cells are loaded with exogenous photosensitizers and most of the ROS are produced in subcellular locations based on the photosensitizer localization (such as plasma membrane, mitochondria or lysosomes) (40). Although ROS can lead to cell death, at minute concentrations they can regulate signal transduction pathways including activation of protein kinases and redox-sensing transcription factors in cardiomyocytes (18).

Light-induced \([\text{Ca}^{2+}]_i\) elevation and its linkage to ROS

It is shown here that visible light at the energy density of 3.6 J/cm\(^2\) induces a small increase in \([\text{Ca}^{2+}]_i\) (Figs. 3b, 7a), which can be mimicked by including low concentrations of \(\text{H}_2\text{O}_2\) to the medium (Figs. 4a, 8a). The question arises of what is the mechanism causing \([\text{Ca}^{2+}]_i\) elevation and how this increase is related to ROS. Neither LDH secretion up to 24 hours after visible light illumination (Fig. 5), nor any morphological damage 24 hours after LEVL or 12 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) treatment was observed (Figs. 6F, 6B). Therefore, the elevation of \([\text{Ca}^{2+}]_i\) cannot be attributed to ROS mediated membrane damage by lipid peroxidation (41) or permeabilization of the membrane to an influx of \(\text{Ca}^{2+}\) (42), as has been previously suggested in PD systems.
The change in \([\text{Ca}^{2+}]_i\) following ROS elevation in un-damaged cells is partially explained by the ability of ROS to mediate direct or indirect phosphorylation of calcium transporters (43), or the ability of ROS to oxidize thiol groups to disulfides in calcium transporters (44, 45). As calcium transporters in muscle cells are highly sensitive to oxidation a oxidation (46, 47). Oxidation of the thiols by ROS can cause structural changes of the transporters, which may inhibit or enhance calcium transport in skeletal and cardiac muscles (44). In the present study, we have found that the increase in \([\text{Ca}^{2+}]_i\) after LEVL illumination or application of 18 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\), is decreased by the voltage dependent calcium channel inhibitor, verapamil (Figs. 7b, 8b), implying that the increase of \([\text{Ca}^{2+}]_i\) after LEVL illumination occurs at least partially via L-type channels. The L-type voltage-gated calcium channels of myocytes were suggested to have a redox sensitive receptor which can be switched on by ROS (48).

The increase of \([\text{Ca}^{2+}]_i\) by L-type channels can cause even a further increase in \([\text{Ca}^{2+}]_i\), by inducing \(\text{Ca}^{2+}\) release from the sarcoplasmic reticulum (SR) (49). Such a mechanism of mobilization of \(\text{Ca}^{2+}\) from internal stores following the initial influx of \(\text{Ca}^{2+}\) through plasma transporters is known as calcium induced calcium released (CICR) mechanism.

**Kinetics of \([\text{Ca}^{2+}]_i\) elevation after illumination or \(\text{H}_2\text{O}_2\) treatment and its correlation to the viability of the cells**

We show in this work that there is a correlation between the kinetics of \([\text{Ca}^{2+}]_i\) elevation following \(\text{H}_2\text{O}_2\) treatment, and the morphology of the cells 24 hours after treatments (Figs. 3, 4 and 6). Such that the \([\text{Ca}^{2+}]_i\) kinetics can indicate the future of the cells 24 hours after treatment. The rapid increase in \([\text{Ca}^{2+}]_i\) (Figs. 3c, 4c) was correlated with an irreversible damage to the cell (Figs 6D, 6G), while the transient increase (Figs. 4a, 3b) was correlated with non-damaged cells. It has been shown that a prolonged increase in \([\text{Ca}^{2+}]_i\), can be lethal, whereas cells can avoid death by using low-amplitude of \([\text{Ca}^{2+}]_i\) signals (50). In photosensitized, illuminated cells, a transient increase in \([\text{Ca}^{2+}]_i\), was suggested to initiate a signal transduction pathway,
contributing to the cell survival (51, 52). Ben-Hur and Penning et al suggested that this transient increase in \([Ca^{2+}]_i\), enabled the cells to accumulate sub-lethal damage (51, 53) by using cellular adaptation responses. This adaptation response includes induction of the transcription and translation of the oxidative stress-related enzyme heme oxygenase (54), induction of heat shock proteins (55), and stimulation of cell growth by an increase in prostaglandin E2 levels (52). Each transient \([Ca^{2+}]_i\) signal is characterized by an amplitude and duration, which are specific in their signaling effects (56). In this study, a treatment with LEVL or a low concentration of H\(_2\)O\(_2\) cause an increase in \([Ca^{2+}]_i\), amplitude of about 12 % , with a duration of more than 60 min. This signal, can be interpreted by the cells as a signal leading to activated different genes (56) and cellular processes (57, 58) that can initiate the ‘healing’ attributed to the therapeutic effect of LEVL.

A known second messenger which regulates \([Ca^{2+}]_i\) signaling is inositol triphosphate (IP\(_3\)) (50, 59). However, in cardiomyocytes the IP\(_3\) receptor is localized at the region of the intercalated discs and little or no IP\(_3\) binding was detected in longitudinal sarcoplasmic reticulum (SR), junctional SR, sarcolemma, mitochondria, and submitochondrial vesicles (60). Moreover, it has been shown that the mass content of IP\(_3\) is lower in isolated cells (which were used in this study) than in the intact tissue (61). Therefore, the IP\(_3\) contribution to the overall calcium homeostasis in cultured cells appears to be negligible (62).

A correlation between the viability of the cells and \([Ca^{2+}]_i\), is not unique to light or ROS as has been shown in this study. Such a correlation was found in gastric cells treated with deoxycholate (63). While high concentrations of the detergent elicit sustained elevation of \([Ca^{2+}]_i\), and were associated with a significant cellular damage, low concentrations of the toxin demonstrated an initial \([Ca^{2+}]_i\) elevation followed by a return towards the resting \([Ca^{2+}]_i\) level and did not appear to induce any cell injury. Moreover, a protective effect against cellular injury induced by a high concentration of the toxin was shown in pretreated cells with a low concentration of the toxin, and
was abolished when the increase in $[\text{Ca}^{2+}]_i$ was prevented by using a $\text{Ca}^{2+}$ chelator (63, 64). This adaptation response which is expressed by preconditioning the cell to a damage, with a mild damage, has many examples (65). Pre-illumination of $E. \text{coli}$ with low intensity visible or IR radiation lead to an increase in cell survival after subsequent irradiations with UV light (66). Pretreatment of several mammalian cell lines with a relatively low concentration of $\text{H}_2\text{O}_2$ increased up to 40-fold their viability after high doses of $\text{H}_2\text{O}_2$ treatment (67).

We conclude that LEVL, by producing ROS such as superoxide anion radicals and $\text{H}_2\text{O}_2$, stimulates a long lasting small increase in $[\text{Ca}^{2+}]_i$ as observed in this study. We speculate that LEVL evokes a cellular adaptation mechanism and calcium signaling, which might explain previously obtained results such as improving functional preservation of isolated rat hearts by low energy photo-irradiation (31), reduction of infarct size of dogs’ hearts (68), and enhancing recovery of ischemic damage in cardiomyocytes (69).
References

1. Blankenship, R. E. (1992) *Photosynth. Res.* **33**, 111
2. Epstein J.M. (1990) *N. Engl. J. Med.* **32**, 1149-1151
3. Conlan, M. J., Rapley, J. W., and Cobb, C. M. (1996) *J. Clin. Periodontol* **23**, 492-496
4. Cohen, N., Lubart R., Rubinstein S., and Breithart, H. (1998) *Photochem. Photobiol.* **68**, 407-413
5. Guzzardella, G. A., Fini, M., Torricelli, P., Giavaresi, G., and Giardino, R. (2002) *Lasers Med. Sci.* **17**, 216-220
6. Kreisler, M., Christoffers, A. B., Al-Haj, H., Willershausen, B., and d'Hoedt, B. (2002) *Laser Surg. Med.* **30**, 365-369
7. Grossman, N., Schneid, N., Reuveni, H., Halevy, S., and Lubart, R. (1998) *Lasers Surg. Med.* **22**, 212-218
8. Yu, W., Naim, J. O., and Lanzafame, R. J. (1995) *FASEB J.* **9**, A239
9. Duan, R., Liu, T. C. Y., Li, Y., Guo, H., and Yao, L.-B. (2001) *Lasers Surg. Med.* **29**, 174-178
10. Spikes, J. D. (1984) *Prog. Clin. Biol. Res.* **170**, 19-39
11. Laloraya, M. M., Pradeep, K. G., and Laloraya M. (1994) *Biochem. Mol. Biol. Int.* **33**, 543-551
12. Cunningham, M. L., Krinsky, N. I., Giovannazzi, S. M., and Peak, M. J. (1985) *J. Free Radic. Biol. Med.* **1**, 381-385
13. Friedmann, H., Lubart, R., Laulicht, I., and Rochkind, S. (1991) *J. Photochem. Photobiol. B Biol.* **11**, 87-95
14. Karu, T. (1999) *J. Photochem. Photobiol. B Biol.* **49**, 1-17
15. Stadler, I., Evans, R., Kolb, B., Naim, J. O., Narayan, V., Buehner, N., and Lanzafame, R. J. (2000) *Lasers Surg. Med.* **27**, 255-261
16. Vanden Hoek, T. L., Becker, L. B., Shao, Z. H., Li, C. G., and Schumacker, P. T. (1998) *J. Biol. Chem.* **273**, 18092-18098
17. Nemoto, S., Takeda, K., Yu, Z. X., Ferrans, V. J., and Finkel, T. (2000) *Mol. Cell. Biol.* **20**, 7311-7318
18. Das, D. K. (2001) *Antioxid. Redox Signal* **3**, 23-37
19. Suzuki, Y. J., and Ford, G. D. (1999) *J. Mol. Cell Cardiol.* **31**, 345-353
20. Oren, D. A., Charney D., Lavie, R., Sinyakov, M., and Lubart, R. (2001) *Biol. Psychiatry* **49**, 464-467
21. Lubart, R., Friedmann, H., and Lavie, R. (2001) Accepted for publication in *Laser Therapy*
22. Lubart, R., Friedmann, H., Sinykov, M., Cohen, N., and Breitbart, H. (1997) *Lasers Surg. Med.* **21**, 1-7
23. Krizaj, D., and Copenhagen, D. R. (2002) *Front Biosci.* **7**, d2023-d2044
24. Kokoska, E. R., Wolff, A. B., Smith, G. S., and Miller, T. A. (2000) *J. Surg. Res.* **88**, 97-103
25. Andrade, F. H., Reid, M. B., Allen, D. G., and Westerblad, H. (1998) *J. Physiol.* **509**, 565-575
26. Lopez-Ongil, S., Torrecillas, G., Perez-Sala, D., Gonzalez-santiago, L., Rodriguez-Puyol, M., and Rodriguez-Puyol, D. (1999) *Free Radic. Biol. Med.* **26**, 501-510
27. Goldman, R., Moshonov, S., and Zor, U. (1998) *Arch. Biochem. Biophys.* **350**, 10-18
28. Sen, C. K. (2000) *Curr. Top. Cell Regul.* **36**, 1-30
29. Livingston, F. R., Lui, E. M., Loeb, G. A., and Forman, H. J. (1992) *Arch. Biochem. Biophys.* **299**, 83-91
30. Specht, K. G., and Rodgers, M. A. J. (2000) *Biochim. Biophys. Acta* **1070**, 60-68
31. Zhu, Q., Yu Wei, X., Hicks, G. L., Ianzafame, R. J., and Wang, T. (1997) *Lasers Surg. Med.* **20**, 332-339
32. Shneyvays, V., Jacobson, K. A., Li, A.-H., Nawrath, H., Zimmerman, T., Isaac, A., and Shainberg, A. (2000) *Exp. Cell Res.* **257**, 111-126
33. Fixler, D., Tirosch, R., Zinman, T., Shainberg, A., and Deutsch, M. (2002) *Cell Calcium* **31**, 279-287
34. Murrant, C. L., and Reid, M. B. (2001) *Microscopy Research and Technique* **55**, 236-248
35. Frejaville, C., Karoui, H., Tuccio, B., Le Moigne, F., Culcasi, M., Pietri, S., Lauricella, R., and Tordo, P. (1995) *J. Med. Chem.* **38**, 258-265
36. Roubaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P., and Zweier, J. L. (1997) *Anal. Biochem.* **247**, 404-411
37. Krishna, M. C., and Samuni, A. (1993) *Free Radic. Biol. Med.* **18**, 239-247
38. Safran, N., Shnevays, V., Balas, N., Jacobson, K. A., Nawrath, H., and Shainberg, A. (2001) *Mol. Cell Biochem.* **217**, 143-152

39. Gomez-Lechon, M. J., O'Connor, E., Castell, J. V., and Jover, R. (2002) *Toxicol. Sci.* **65**, 299-308

40. Moor, A. C. (2000) *J. Photochem. Photobiol. B Biol.* **57**, 1-13

41. Tarr, M., Frolov, A., and Valenzeno, D. P. (2001) *Photochem. Photobiol.* **73**, 418-424

42. Yonuschot, G., Vaughn, J. M., and Novotny, J. F. (1992) *J. Mol. Cell Cardiol.* **24**, 1079-1088

43. Rothstein, E. C., Byron, K. L., Reed, R. E., Fliegel, L., and Lucchesi, P. A. (2002) *Am. J. Physiol. Heart Circ. Physiol.* **283**, H598-H605

44. Anzai, K., Ogawa, K., Ozawa, T., and Yamamoto, H. (2000) *Antioxid. Redox Signal* **2**, 35-40

45. Wang, H., and Joseph, J. A. (2000) *Free Radic. Biol. Med.* **28**, 1222-1231

46. Dulhunty, A., Haarmann, C., Green, D., and Hart, J. (2000) *Antioxid. Redox Signal* **2**, 27-34

47. Yamaoka, K., Yakehiro, M., Yuki, T., Fujii, H., and Seyama, I. (2000) *Pflugers Arch.* **440**, 207-215

48. Campbell, D. L., Stamler J.S., and Strauss H.C. (1996) *J. Gen. Physiol.* **108**, 277-293

49. Wang, S. Q., Song, L. S., Lakatta, E. G., and Cheng, H. (2001) *Nature* **410**, 592-596

50. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) *Nature* **395**, 645-648

51. Penning, L. C., Rasch, M. H., Ben-Hur, E., Dubbelman, T. M., Havelaar, A. C., Van der Zee, J., and Van Steveninck, J. (1992) *Biochim. Biophys. Acta* **1107**, 255-260

52. Penning, L. C., Keirse, M. J., Van Steveninck, J., and Dubbelman, T. M. (1993) *Biochem. J.* **292**, 237-240

53. Ben-Hur, E., and Dubbelman, T. M. A. R. (1993) *Photochem. Photobiol.* **58**, 890-894

54. Ben-Hur, E., Dubbelman, T. M. A. R., and Van Steveninck, J. (1991) *Photochem. Photobiol.* **54**, 163-166

55. Fisher, A. M. R., Ferrario, A., and Gomar, C. J. (2002) *Photochem. Photobiol.* **58**, 581-588

56. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997) *Nature* **386**, 855-858

57. Sauer, H., Diedershagen, H., Hescheler, J., and Wartenberg, M. (1997) *FEBS Lett.* **419**, 201-205

58. Wartenberg, M., Hescheler, J., and Sauer, H. (1997) *Am. J. Physiol.* **272**, R1677-R1683
59. Berridge, M. J., and Irvine, R. F. (1989) *Nature* **341**, 197-205

60. Kijima, Y., Saito, A., Jetton, T. L., Magnuson, M. A., and Fleischer, S. (1993) *J. Biol. Chem.* **268**, 3499-3506

61. Fitzgerald, M., Anderson, K. E., and Woodcock, E. A. (1994) *Eur. J. Pharmacol.* **268**, 275-278

62. Zucchi, R., Ronca, F., and Ronca-Testoni, S. (2001) *Pharmacol. Ther.* **89**, 47-65

63. Kokoska, E. R., Smith, G. S., Wolff, A. B., Deshpande, Y., Rieckenberg, C. L., Banan, A., and Miller, T. A. (1998) *Am. J. Physiol.* **275**, G322-G330

64. Miller, T. A., Kokoska, E. R., Smith, G. S., and Banan, A. (2001) *Life Sci.* **69**, 3091-3102

65. Crawford, D. R., and Davies, K. J. (1994) *Environ. Health Perspect.* **102 Suppl 10**, 28

66. Lage, C., Teixeira, P. C., and Leitao, A. C. (2000) *J. Photochem. Photobiol. B Biol.* **54**, 155-161

67. Wiese, A. G., Pacifici, R. E., and Davies, K. J. (1995) *Arch. Biochem. Biophys.* **318**, 231-240

68. Oron, U. (2001) *Circulation* **103**, 269-301

69. Yaakov, N., Bdolah, A., Wollberg, Z., Ben-Haim, S. A., and Oron, U. (2000) *Basic Res. Cardiol.* **95**, 385-388
Footnote

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

Figure 1: Light induced H$_2$O$_2$ formation in cardiomyocytes. Luminol luminescence from cardiomyocyte suspension illuminated with 3.6 J/cm$^2$ visible light compared to that of control (non-illuminated) cells. $p \leq 0.047$ using the Anova single factor test (*).

Figure 2: EPR spin trapping measurements of super oxide anion with the spin trap, DEPMPO. (a) Simulated spectrum of the superoxide adduct of DEPMPO (DEPMPO-OOH), (assuming two conformers, A and B, of DEPMPO-OOH with the EPR hyperfine splitting constant (36): A (43%): $a_N = 13.13$ G; $a_p = 55.61$G; $a^\beta_H = 13.11$G; $a^\gamma_H = 0.71, 0.60, 0.25, 0.42,$ and 0.70 G. B (57%): $a_N = 13.08$G; $a_p = 45.85$G; $a^\beta_H = 9.53$G; $a^\gamma_H = 1.05, 0.7, 0.25, 0.42$ and 0.6 G; (b) EPR spectrum of $10^7$ cardiomyocyte cells/ml + 0.02 M DEPMPO during illumination; (c) EPR spectrum of $10^7$ cells/ml + 0.02 M DEPMPO without illumination; (d) EPR spectrum of $10^7$ cardiomyocyte cells/ml + 0.02 M DEPMPO + SOD (100 U /ml) during illumination. The asterisk shows lines corresponding to the adduct superoxide.

Figure 3: Effect of visible light (40 mW/cm$^2$) on [Ca$^{2+}$]$_i$ in 3-4 day old cardiomyocytes. The cells were illuminated as follows: (a - □) no illumination; (b - ▲) 1.5 min illumination (3.6 J/cm$^2$); (c - ○) 5 min illumination (12 J/cm$^2$); (d - ∆) 5 min illumination in the presence of 200 U/ml of catalase. Each curve represents an average of 3-6 different experiments (light was applied at time 0).

Figure 4: Effect of different concentrations of hydrogen peroxide on [Ca$^{2+}$]$_i$ in 3-4 day old cardiomyocytes. (a - ▲) 12 µM H$_2$O$_2$ (also shown in small window); (b - ◊) 24 µM H$_2$O$_2$; (c - ○) 48 µM H$_2$O$_2$; (d - □) no treatment. Each curve represents an average of 3-6 different experiments (H$_2$O$_2$ was applied at time 0).
Figure 5: Release of LDH to the medium of illuminated cardiomyocytes. The cells were treated with 3.6J/cm² or 12 J/cm² illumination and LDH was measured immediately and after: 2.5 hours; 6 hours; or 24 hours after treatment.

Figure 6: Light microscopy of 4 day old cardiac cells, stained with desmin antibodies and hematoxyline (scale: bar=10 µM). (A) control cells; (B) cells exposed to H₂O₂, 12 µM; (C) cells exposed to H₂O₂ 24 µM; (D) cells exposed to H₂O₂ 48 µM; (E) light illuminated cells, 1.5 min illumination (3.6 J/cm²); (F) light illuminated cells, 3 min illumination (7.2 J/cm²); (G) light illuminated cells, 5 min illumination (12 J/cm²); (H) light illuminated cells, 5 min (12 J/cm²), in the presence of catalase (200 U/ml). Picnotic damage to the nuclei are shown by the white arrows and signs of perinuclear edema are shown by blue arrows.

Figure 7: Effect of visible light on [Ca²⁺]i in cardiomyocytes treated in the presence of verapamil. (a - ▲) After 1.5 min illumination (3.6 J/cm²); (b - ∆) After 1.5 min illumination in the presence of 10 µM verapamil; (c - □) In the presence of 10 µM verapamil without illumination. Each curve represents an average of 3-6 different experiments. (Light was applied at time 0).

Figure 8: Effect of hydrogen peroxide on [Ca²⁺]i in cardiomyocytes in the presence of verapamil. (a - ▲) 18 µM H₂O₂ without verapamil; (b - ∆) In the presence of 18 µM H₂O₂ and 10 µM verapamil. (c - □) In the presence of 10 µM verapamil without H₂O₂. Each curve represents an average of 3-6 different experiments. (H₂O₂ was applied at time 0).
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Low energy visible light induces reactive oxygen species generation and stimulates an increase of intracellular calcium concentration in cardiac cells
Ronit Lavi, Asher Shainberg, Harry Friedmann, Vladimir Shnevays, Ophra Rikover, Maor Eichler, Doron Kaplan and Rachel Lubart

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