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Singlet Oxygen and ROS in a New Light: Low-Dose Subcellular Photodynamic Treatment Enhances Proliferation at the Single Cell Level

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Abstract

Two-photon excitation of a sensitizer with a focused laser beam was used to create a spatially-localized subcellular population of reactive oxygen species, ROS, in single HeLa cells. The sensitizer used was protoporphyrin IX, PpIX, endogenously derived from 5-aminolevulinic acid delivered to the cells. Although we infer that singlet oxygen, $O_2(a^1\Delta_g)$, is one ROS produced upon irradiation of PpIX under these conditions, it is possible that the superoxide ion, $O_2^{-\cdot}$, may also play a role in this system. With a "high" dose of PpIX-sensitized ROS, the expected death of the cell was observed. However, under "low dose" conditions, clear signs of cell proliferation were observed. The present results facilitate studies of ROS-mediated signalling in imaging-based single cell experiments.

Introduction

The photosensitized production of singlet molecular oxygen, $O_2(a^1\Delta_g)$, in or near a cell has long been recognized as an event that results in the death of that cell.[1, 2] This phenomenon has been exploited in Photodynamic Therapy, PDT, a treatment methodology wherein $O_2(a^1\Delta_g)$ is used to destroy cancer cells, for example. Although the underlying mechanisms of PDT have been extensively studied for over 30 years, there is much still to be learned about the exact role(s) played by $O_2(a^1\Delta_g)$ in the often complicated processes that can result in cell death.[2, 3] This statement is also pertinent with respect to other reactive oxygen species, ROS, which might be concurrently formed in the photosensitized process. A relevant example in this regard is the superoxide ion, $O_2^{\cdot-}$, that is formed as a consequence of a photoinitiated electron transfer reaction.[3, 4]

Recent developments in laser-based optical methods and spectroscopic techniques, combined with a better understanding of the photophysics and photochemistry of $O_2(a^1\Delta_g)$ produced in a photosensitized process, have provided increasingly informative pictures of the behavior of $O_2(a^1\Delta_g)$ in biological systems.[5, 6] We have been particularly interested in developing and exploiting techniques whereby $O_2(a^1\Delta_g)$ can be selectively produced, detected and studied in single cell experiments.[5, 7, 8] The advent of near IR fs lasers has been a wonderful asset for sub-micrometer spatially-resolved studies in cells,[9] and we have used this tool for the two-photon excitation of a sensitizer allowing accurate control of the cytotoxic $O_2(a^1\Delta_g)$ "dose" delivered in or near a cell.[10-13]

The tools and methods we have developed allow us to uniquely address a specific, and arguably controversial, photoinduced oxygen-dependent phenomenon that appears to be the antithesis of ROS-mediated necrotic/apoptotic cell death. Low Level Light Therapy (LLLT) refers to diverse cell-stimulating phenomena that occur as a result of irradiating cells and tissue with very

low doses of light.[14-17] Unfortunately, many LLLT experiments are performed without accurate control over pertinent variables and this, in turn, leads to confusion over the mechanistic events that occur.[14, 16-18] In this Communication, we report that, in single cell photosensitized experiments, controlled low doses of intracellular ROS are not cytotoxic. Rather, the ROS produced stimulate the cell in such a way that mitosis occurs more rapidly. We thus present a tool that facilitates control over selected experimental variables which, in turn, can contribute to better elucidating mechanistic aspects of a broad spectrum of events, ranging from cell proliferation to cell death, in which ROS-mediated cell signaling plays a role.

Results and discussion

High dose versus low dose

In earlier "high dose" studies, we demonstrated that spatially-confined two-photon excitation of an intracellular sensitizer, protoporphyrin IX (PpIX), gave rise to morphological signs characteristic of cell death.[11, 12] For these experiments, PpIX was incorporated into the cell by incubating the cell with 5-aminolevulinic acid (ALA) which is a precursor in the biosynthesis of PpIX. The final stages of this synthesis occur in the mitochondria and, as such, PpIX tends to first localize in this organelle.[19-21] The latter can be verified by recording images of the cell based on PpIX fluorescence.[19]

In the context of our present Communication, these earlier experiments allow us to characterize a cytotoxic "dose" of intracellular ROS sensitized by the focused two-photon irradiation of PpIX. Specifically, morphological signs of death were recorded from HeLa cells upon pulsed fs laser irradiation of intracellular PpIX at 800 nm for 30 min (80 MHz repetition rate, laser beam diameter at the cell of ~ 1 μm, temporal pulse width < 100 fs, 2.2 mW average power

measured at the cell on the microscope stage).[12] Note that, although 3.96 J were delivered to the cell during the irradiation period under these conditions, only a fraction of this energy was absorbed by PpIX in the two-photon process. With this approach and the data thus recorded as a reference, we then set out to monitor cell response to a much lower dose of PpIX-sensitized ROS in corresponding single cell experiments.

For the present study, HeLa cells were seeded at a low density so that each cell was isolated from neighboring cells. Because we are assessing cell response in terms of a photo-mediated change in the rate at which the cells undergo mitosis, it is important to ensure that the cells under study have cell cycles that, in the absence of a perturbation, are synchronized. We exploited the fact that when a cell divides, the resultant two "sister" cells will have cell cycles that are synchronized (*i.e.*, synchronized because they derive from the same progenitor cell). Thus, our experiments were performed once the originally-seeded cells had divided for the first time. In this way, we avoided cell synchronization by stressful conditions, like serum deprivation or chemical compound exposure. Of the twin sister cells that resulted from this first division, one was exposed to the laser and the other was left as a control. For experiments involving the presence of intracellular PpIX, ALA was present in the culturing medium (1 mM ALA for 4 h prior to laser exposure).

The laser-treated cell was irradiated at 800 nm with the same 80 MHz fs laser used in our previous cell-kill studies.[12] For the present experiments, however, the irradiation period was only 30 s at an average laser power of 4.8 mW corresponding to 0.14 J delivered to the cell. The latter was measured at the focal plane on the microscope stage. The focused ~ 1 μm diameter laser beam was positioned at a place in the cytoplasm where the local PpIX concentration was high (as judged by independent images based on PpIX fluorescence, see Figure 1). The pair of sister cells was then periodically monitored for up to 18 h using bright field images (the wavelength used was not

absorbed by PpIX), and we noted when each cell in the pair started mitosis. Examples of images thus obtained are shown in Figure 2.

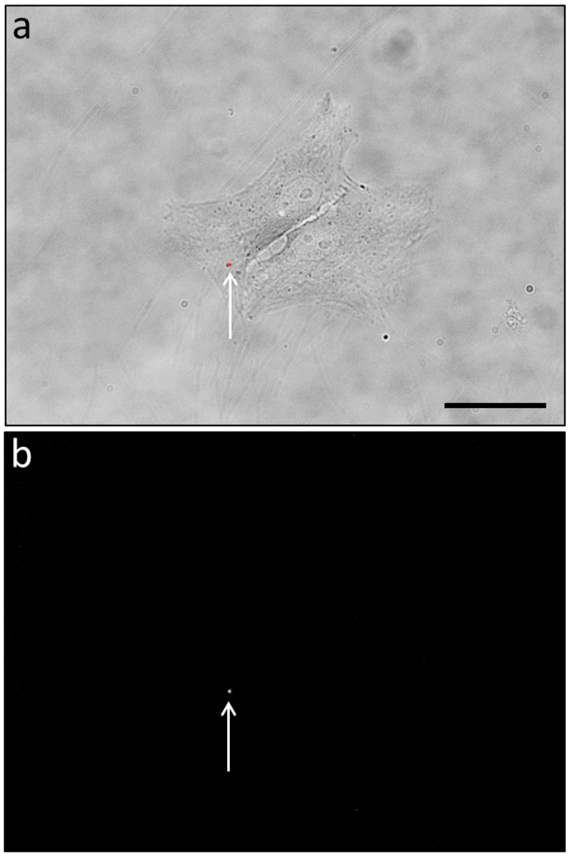

Figure 1. (a) Bright field image of sister cells containing PpIX. The cell to the left was irradiated with the focused laser used to generate ROS. The location and size of the domain of PpIX excited states created by the two-photon process, as ascertained from a PpIX fluorescence image (panel b), is indicated by the red dot (white arrow). Scale bar = 20 μm. (b) Image of the same two cells based on PpIX fluorescence excited by the focused actinic laser pulse.

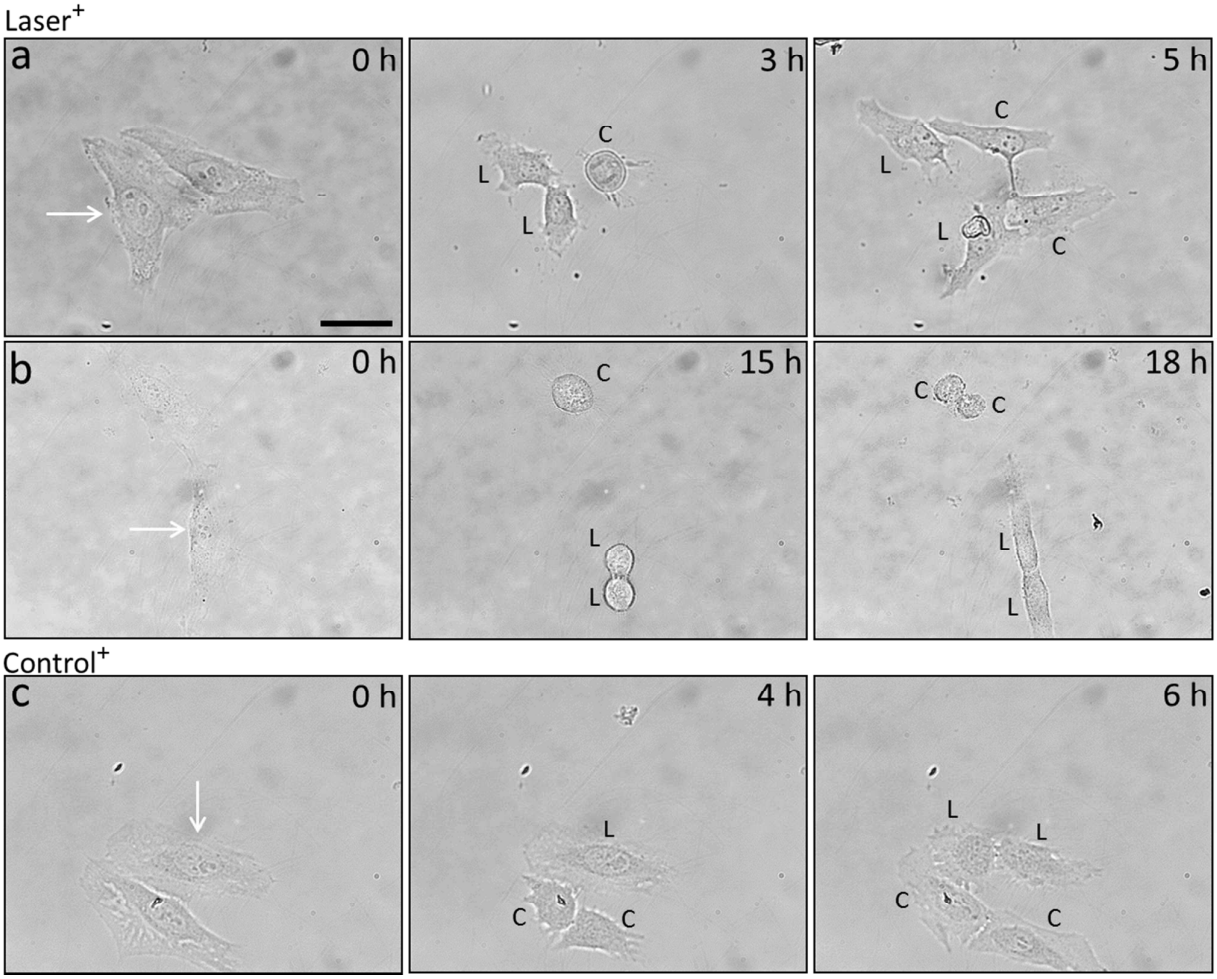

Figure 2. Bright field images of sister HeLa cells whose cell cycles, before irradiation, are synchronized. For all cases shown, the cells had been incubated with ALA for 4 h prior to irradiation. (a and b) Examples of "Laser^{+}" where the irradiated cell undergoes mitosis before the un-irradiated control cell. (c) Examples of "Control^{+}" where the un-irradiated control cell undergoes mitosis before the irradiated cell. The irradiated cell is indicated with a white arrow and its daughter cells labelled with the letter "L", whereas the un-irradiated control cell and its daughters are labelled with a "C". For each experiment, data are shown for different times after the 30 s laser exposure. Scale bar = 20 μm

If a laser-exposed cell started mitosis at least 2 h before its un-irradiated control partner then the response was evaluated as "Laser^{+}". When the control cell proliferated at least 2 h before the laser-exposed cell it was evaluated as "Control^{+}". If both cells started mitosis within 2 h of each other, the response was considered "Equal". The 2 h period was established because images used to assess cell response were taken outside the cell incubator. After imaging, cells were returned to the incubator. Two hours was judged to be a reasonable compromise between the time for imaging and usual conditions for equilibration with the culturing medium. Our choice in this regard was shown to be acceptable because control cultures exposed to the same conditions did not show deleterious effects.

For the experiments involving PpIX, 97 independent sets of sister cells were examined. Of these, 16 sister cells did not start mitosis within the 18 h observation period. Thus, the Laser^{+}/Equal/Control^{+} evaluation was made using 81 pairs of sister cells. Likewise, 61 pairs of sister cells lacking PpIX were examined and, of these, 5 pairs did not start mitosis within the 18 h observation period. Thus, here the Laser^{+}/Equal/Control^{+} evaluation was made using 56 pairs of sister cells.

As illustrated in Figure 2 for cells containing PpIX, both the control and laser-exposed cells always showed normal mitotic behavior, irrespective of the order in which they entered the mitotic phase. Although not shown, the same was also observed for cells without added PpIX. These are important observations; they indicate that the laser treatment is not harmful. If laser exposure had adversely influenced the cells, these cells would have transiently arrested their mitotic response in order to repair the damage.[16, 22] Alternatively, these cells would simply have aborted proliferation, either entering into a senescence-like state or starting their apoptotic program.[16, 23] In support of these observations, there was no morphological evidence characteristic of cell damage for any of the irradiated cells over our 18 h observation period after irradiation.

The frequency of the respective cell responses is shown in Figure 3. An important conclusion can be drawn when considering the data recorded from cells containing PpIX (histogram to the right in Figure 3). It is clear that the irradiated cells, and hence the cells in which PpIX-sensitized ROS were produced, have a greater tendency to start mitosis well before the un-irradiated cells (*i.e.*, the "Laser$^+$" category is significantly larger than the "Control$^+$" category; $p = 0.015$). Recall that our threshold for discriminating between the Laser$^+$ and Control$^+$ categories is a time difference of 2 h. The histogram to the left shows the results from cells that had not been incubated with ALA and, hence, did not contain added PpIX. The laser-exposed cells in this group proliferate at the same rate as their control un-irradiated sister cells.

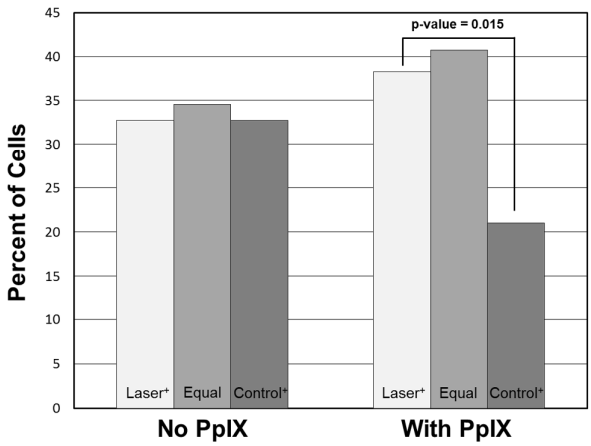

Figure 3. Percentages of a given cell population categorized according to their mitotic rate response to irradiation. For the system without PpIX, the data shown are based on 56 pairs of sister cells. For the case with PpIX, the data shown are based on 81 pairs of sister cells.

The data in Figure 3 also allow us to eliminate local heating effects as a cause of the observed cell proliferation. At the irradiation wavelength of 800 nm, one-photon overtone absorption by water in the cell, and the subsequent dissipation of this energy as heat, effectively competes with the two-photon electronic transition in PpIX. Thus, the combination of (1) the

absence of selective laser-induced proliferation in cells lacking PpIX, and (2) the inference that most of the photons absorbed by PpIX result in ROS formation, *vide infra*, indicate that our data are consequence of something other than local laser-induced heating.

Quantifying the amount of ROS produced

It is important to note that we are currently limited to performing our present experiment only in relation to a corresponding experiment that results in cell death; we are still not able to accurately quantify the absolute concentration of PpIX-sensitized intracellular ROS produced in a given single cell experiment. Although the concentration of $O_2(a^1\Delta_g)$, for example, can be obtained in analogous experiments performed upon irradiation of an extracellular sensitizer,[10] there are still too many undefined parameters to provide a sufficiently accurate absolute value of the $O_2(a^1\Delta_g)$ concentration in our intracellular irradiation volume. Nevertheless, we are still able to make some qualitative statements that are pertinent in this regard. For the experiments performed on cells containing PpIX, the same ALA incubating conditions and irradiating laser powers were always used. Thus, one might infer that the same amount of ROS was always generated upon irradiating a given cell containing PpIX. Unfortunately, even this inference is not really justified given, for example, that different amounts of PpIX can be made in a cell despite identical incubating conditions with ALA.[19] Thus, to further solidify our arguments, we set out to quantify the relative amounts of excited-state PpIX made in each of the cells irradiated. To accomplish this we monitored the intensity of PpIX fluorescence produced by our actinic laser pulse, integrating the data obtained over the 30 s irradiating period. Although there are also limitations to this approach as a method to accurately quantify the dose of ROS produced,[11, 12, 24, 25] it should nevertheless provide an important qualitative warning if something is wrong with our mitosis data shown in Figure 3.

An example of the spatially-localized two-photon-excited PpIX fluorescence signal monitored for these experiments is shown in Figure 1. The PpIX fluorescence data thus obtained from these irradiated cells was then subdivided according to whether, within the pertinent pair of sister cells, the irradiated cell initiated mitosis first (Laser$^+$), the un-irradiated cell initiated mitosis first (Control$^+$), or the sister cells initiated mitosis at the same time (Equal). The results are shown in the histogram on the right side of Figure 4. Admittedly, the standard deviations on these data sets are large. This partially reflects the fact that the intensity of the light monitored is very low; *i.e.*, the quantum yield of PpIX fluorescence is small ($\Phi_f \sim 0.02$ - 0.04),[26] the two-photon absorption cross section of PpIX is small ($\delta \sim 2 \times 10^{-50}$ cm^4 s photon^{-1} at 790 nm),[27] and the integrated laser energy used for excitation is small (4.8 mW over 30 s). Nevertheless, it is reassuring to see that irrespective of whether the mitotic response was Laser$^+$, Equal, or Control$^+$, it appears that the same amount of PpIX was always excited in the irradiated cell. In short, these data do not invalidate the result shown in Figure 3 that is statistically significant. (Considering the way the Laser$^+$ and Control$^+$ data were recorded for Figure 3, we can only provide the more appropriate *p* value obtained through the "non-directional *z* hypothesis test"; error bars based on a standard deviation have no statistical meaning in this case.[28, 29]) Also shown in Figure 4 is the intensity of the emission signal recorded upon focussed 800 nm irradiation of cells that were not incubated with ALA, subdivided according to the same criteria. The non-zero signals recorded in this latter case likely reflect, in part, emission from endogenous fluorophores.

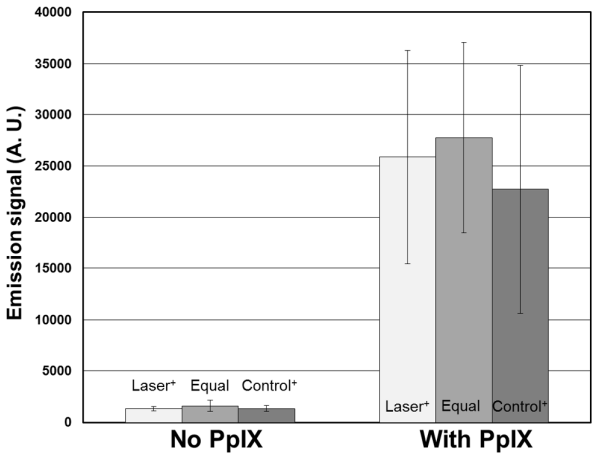

Figure 4. Integrated emission intensities observed upon two-photon irradiation of HeLa "sister" cells at 800 nm. The data are subdivided according to whether, within the pertinent pair of synchronized sister cells, the irradiated cell initiated mitosis first ($Laser^{+}$), the un-irradiated cell initiated mitosis first ($Control^{+}$), or the sister cells initiated mitosis at the same time (Equal). Emission intensities represent values that were integrated over the actinic irradiation period of 30 s. Error bars denote one standard deviation.

Singlet oxygen and/or the superoxide ion

On the basis of experiments performed in bulk solvents, PpIX is known to be a reasonably good $O_2(a^1\Delta_g)$ sensitizer ($O_2(a^1\Delta_g)$ quantum yield, ϕ_Δ, of ~0.6).[30] It is generally inferred that intracellular PpIX is likewise a good $O_2(a^1\Delta_g)$ sensitizer. However, there is sufficient precedence for a host of sensitizers that photo-initiated electron transfer reactions that ultimately produce the superoxide ion, $O_2^{-\cdot}$, can efficiently compete with $O_2(a^1\Delta_g)$ production when that sensitizer is incorporated in a protein-rich environment (*e.g.*, data recorded from flavin mononucleotide[31] and phenalenone[32]). Evidence to this effect has likewise been presented for PpIX, although given the experimental protocol used in that study it is uncertain whether the superoxide produced is formed directly upon quenching of the PpIX excited state by oxygen or as a consequence of secondary and

tertiary reactions mediated by other reaction intermediates, including $O_2(a^1\Delta_g)$.[33] In any event, more experiments will be required to ascertain exactly what ROS are involved in the intracellular PpIX-sensitized nascent events that determine the longer-term ramifications of proliferation and/or cell death.

Mechanistic considerations

It now remains to explain the phenomenon that we have documented under this controlled set of conditions. In doing so, it will clearly be important to consider aspects of our present work in the context of other ROS-mediated cell proliferation studies.[14-16, 23] Unfortunately, many pertinent answers are not available, and getting them will require a continued long-term effort from the research community at large. At present, we can only pose a few questions and suggestions that might drive future experiments. For example, are the initial dose-dependent targets of $O_2(a^1\Delta_g)$ in a cell that lead either to cell death or cell proliferation the same? If they are the same, is the observed increased rate of mitosis still a stress response and/or part of a repair mechanism to counteract the "damaging" effects of $O_2(a^1\Delta_g)$? Also, considering that the ROS dose delivered to each cell in our study was identical, it is possible that the difference between the Laser$^+$ and Control$^+$ responses could be that one pair of sister cells was perturbed at a point in its cell cycle which was different from that in another pair independently perturbed (*i.e.*, the specific phase of the cell cycle at the moment of the photodynamic treatment may be of importance in deciding the cell response).[23, 34, 35] Finally, it will be interesting to see if this phenomenon is also observed for low doses of extracellular ROS. In this latter case, we can exert greater control in the production of $O_2(a^1\Delta_g)$ at the expense of the superoxide ion.[10]

Conclusions

In summary, using fs laser-based subcellular-localized irradiation, we have been able to significantly increase the proliferation rate of HeLa cells through a sensitized photodynamic treatment that produces a low dose of ROS and, consequently, a low dose of $O_2(a^1\Delta_g)$. None of the cells in which ROS were produced showed signs of cytotoxic damage typically associated with higher doses of ROS.[12] Specifically, all cells studied proceeded through the mitotic cell cycle without problem. To our knowledge, such a ROS-mediated increase in cell proliferation has not previously been observed in experiments performed at the single cell level. The tools and methodology presented in this Communication should be useful in studies to further elucidate issues relating to ROS- and $O_2(a^1\Delta_g)$-mediated cell signalling.

Acknowledgements

This work was supported by grants from the Danish National Research Foundation and the European Molecular Biology Organization, EMBO (ASTF 22-2013). The authors thank Puk Lund for assistance with cell culturing.

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