Prolonged lipid oxidation after photodynamic treatment. Study with oxidation-sensitive probe C11-BODIPY$^{581/591}$

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Abstract Photodynamic treatment (PDT) is an emerging procedure for the therapy of cancer, based on photosensitizers, compounds that generate highly reactive oxygen species on illumination with visible light. Photodynamic peroxidation of cellular lipids is a consequence of PDT associated with cytolethality. We used chloromethyl dichlorodihydrofluorescein diacetate and a novel fluorescent ratiometric oxidation-sensitive probe, C11-BODIPY$^{581/591}$ (C11-BO), which reports on lipid peroxidation, for visualizing oxidative stress in cells subjected to PDT with a phthalocyanine photosensitizer Pc4. With C11-BO loaded into the cells before or immediately after PDT, we observed a prolonged oxidation, which continued up to 30 min after illumination. In contrast, H_2O_2 caused oxidation of C11-BO only when the cells were in direct contact with H_2O_2. PDT-induced oxidative stress was most pronounced in vesicular perinuclear organelles, most likely photodamaged lysosomes. We hypothesize that the lysosomal localization of the prolonged oxidative stress is a consequence of the presence of redox-active iron in lysosomes. In conclusion, we have found that oxidative stress induced in cells by PDT differs from one induced by H_2O_2 in respect of induction of prolonged oxidation of lipids.

Keywords: Confocal fluorescence microscopy; Living cells; Lipid peroxidation; Oxidation-sensitive probes; Oxidative stress; Photodynamic treatment

1. Introduction

Photodynamic therapy is an emerging modality for treatment of cancer [1,2]. The photodynamic treatment (PDT) includes loading of the target cells with a photosensitizer and subsequent illumination with visible light. In the presence of oxygen, the combination of light and photosensitizer causes generation of reactive oxygen species (ROS), in particular singlet oxygen, superoxide anion and hydroxyl radical [3], resulting in target cell death either through necrosis or apoptosis [4–6]. The most active ROS generated by PDT have very short lifetimes (less than 1 μs) in the intracellular environment [3].

Different photosensitizers have different intracellular localizations. Most of them are hydrophobic substances, which may localize to plasma membrane and various cytoplasmic membranes. Due to short lifetimes of the ROS generated, their radius of action is limited. As a result, primary oxidative damage induced by PDT localizes to different intracellular compartments depending on the localization of a particular photosensitizer [3,4].

In recent years, a lot of progress has been made in the identification of signaling pathways activated by PDT and the characterization of the mechanisms of cellular death induced by PDT [4–6]. However, the knowledge of primary targets of PDT-induced oxidation and intracellular localization of the primary oxidative stress is lacking. For the proper characterization of PDT with any particular photosensitizer, one needs to identify the molecular targets, which are affected as a result of oxidative stress produced by PDT. It is also important to find out in which organelles the oxidative stress is induced during PDT and how this initial oxidative stress propagates within the cell.

In our previous paper, we have characterized the proteins susceptible to PDT-induced oxidation in living cells [7]. In the present study, we applied oxidation-sensitive fluorescent probes to visualize the intracellular localization and to assess the time course of oxidative stress, triggered by PDT. For PDT, we used a phthalocyanine compound Pc4, a highly efficient photosensitizer known to localize to several intracellular compartments including mitochondria, lysosomes and Golgi apparatus [8].

2. Materials and methods

2.1. Materials

C11-BODIPY$^{581/591}$ (C11-BO), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCF), Mitotracker Red, Acridine Orange (AO), Hoechst 33342 and propidium iodide were from Molecular Probes (Leiden, The Netherlands). Photosensitizer Pc4 was kindly provided by Dr. N. Oleinick from Case Western Reserve University, Cleveland.

2.2. Cell culture and photodynamic treatment

Rat-1 fibroblasts were cultured in DMEM with 7.5% fetal calf serum. The experiments were performed with 70–80% confluent cells growing in glass-bottomed 3.5-cm dishes (Willco Wells, Amsterdam, The Netherlands). Cells were loaded with Pc4 in the culture medium without serum for 3 h. After washing away the photosensitizer, the cells were illuminated for 30 s with visible light to reach the fluence of 0.24 J/cm^2 (fluence rate 8 mW/cm^2). Slide projector equipped with a 250 W tungsten lamp was used as a light source. The part of the light

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Abbreviations: PDT, photodynamic treatment; ROS, reactive oxygen species; C11-BO, C11-BODIPY$^{581/591}$; CM-DCF, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; AO, Acridine Orange

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spectrum with $\lambda < 470$ nm was cut off by a short-cut filter. The fluence rate was measured with an optical power meter Advantest TQ8210. Combined Hoechst 33342/propidium iodide staining (both at 2 μg/ml in the culture medium) was used to detect dead and apoptotic cells. Apoptotic cells were identified by characteristic morphology (blebbing) and by nuclear condensation/fragmentation revealed with Hoechst 33342 staining.

2.3. Oxidation-sensitive probes and organelle staining

Oxidation-sensitive fluorescent probes were loaded into the cells for 15 min immediately before or immediately after illumination. Oxidation of the probes was documented with confocal laser scanning microscopy performed using a Nikon Eclipse TE2000-U microscope, equipped with confocal C1 unit. Green fluorescence was excited with 488 nm line of Argon–Ion laser and detected with emission bandpass filters 515/30, red fluorescence with 543 nm line of He–Ne laser and 585/30 emission filter, infra-red fluorescence with 633 nm line of He–Ne laser and longpass 665 nm emission filter.

Two oxidation-sensitive fluorescent probes were used. (1) Fluorescent fatty acid C11-BO is a ratiometric probe, which reports on the oxidation of lipids in living cells [9,10]. Upon reaction with radicals, the red fluorescence of this fluorophore shifts to green. The stock solution of the probe was prepared by dissolving the probe in fetal calf serum at 20 μM. For loading the cells, this stock was diluted in DMEM to a final concentration of 1 μM and incubated with cells for 15 min at 37 °C. The green and red fluorescence of C11-BO was acquired using double wavelength excitation and detection. (2) CM-DCF is a non-ratiometric probe sensitive to a wide range of radicals [11]. Initially non-fluorescent, the probe produces green fluorescence upon oxidation. The probe was dissolved in DMSO at 1 mM shortly before each experiment and was loaded into the cells for 15 min at 37 °C at a final concentration of 2 μM.

Pc4 was visualized using infra-red fluorescence settings. To visualize mitochondria and lysosomes, cells were stained for 5 min with Mitotracker Red and AO at 100 nM. Both probes fluoresce red inside mitochondria and lysosomes, respectively. In addition, AO gives a weak green fluorescence in cytoplasm and a stronger green fluorescence in the nucleus.

2.4. Ratio imaging of C11-BO. Calculation of the degree of oxidation of C11-BO in the cells

To calibrate photomultipliers of the green and red channels, the images of intact cells loaded with C11-BO were compared to images of the same cells after complete photooxidation of C11-BO achieved

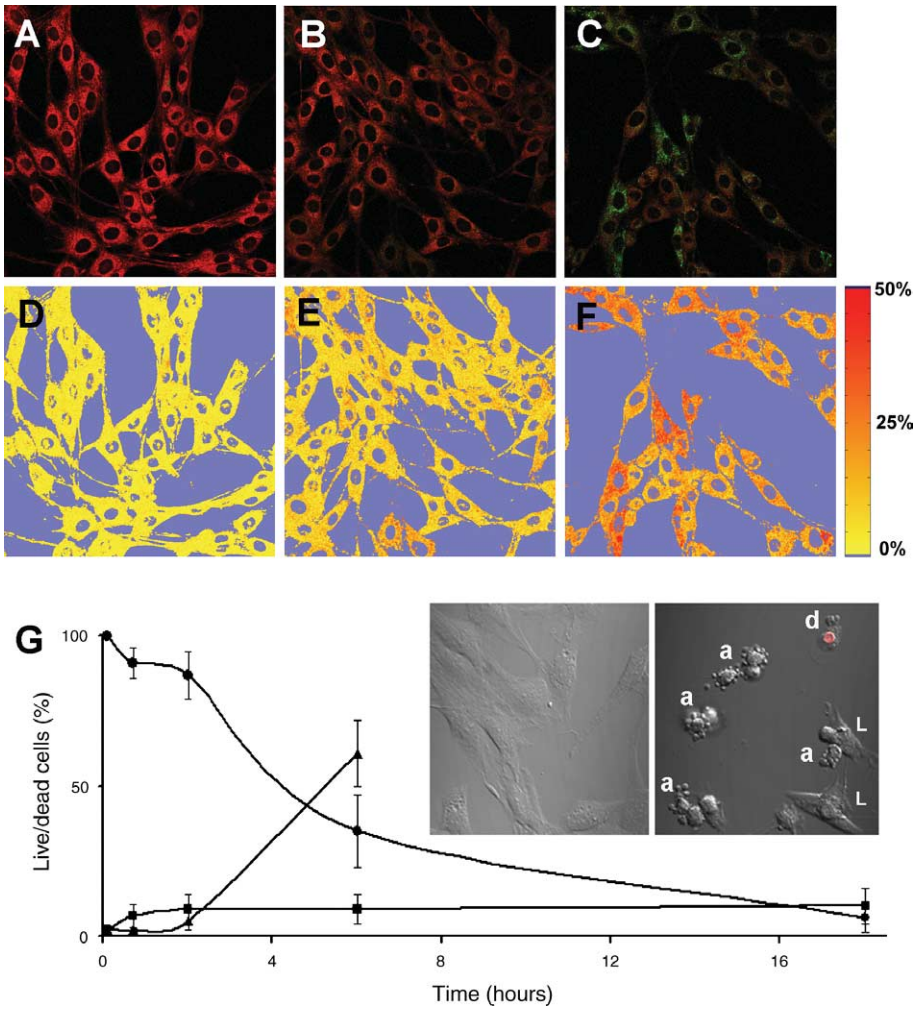

Fig. 1. Oxidation of C11-BO and cell death after PDT. Cells were loaded with Pc4, then with C11-BO, illuminated, and images were taken with the confocal microscope. (A) Before illumination; (B) 1 min after illumination; (C) 15 min after illumination. C11-BO oxidation manifests in conversion of red fluorescence to green. (D, E, F) Results of ratio imaging of panels A, B and C, respectively. The degree of the probe oxidation is presented as gradations between yellow for 0% oxidation and red for 50% oxidation (see the colorbar next to panel F). (G) Cell death after PDT. ●, live cells; ■, dead (necrotic) cells; ▲, apoptotic cells. Apoptotic cells could not be counted accurately at 18 h because of high degree of decomposition. Inserts show the morphology of control (no photosensitizer) cells (left) and Pc4-loaded cells (right) 6 h after illumination. Letters in the right insert stand for: *d*, dead (necrotic) cell with the nucleus stained with propidium iodide, *a*, apoptotic cells; *L*, live cells.

by 30 s exposure to light from the mercury lamp of the microscope. Such exposure caused almost quantitative conversion of non-oxidized probe (red) into oxidized one (green). Gains of the two photomultipliers were adjusted to achieve equal signal in red channel before photooxidation and in green channel after photooxidation.

Cells loaded with C11-BO and subjected to various forms of oxidative stress were imaged in three different microscopic fields for each time point. Images were analyzed with a custom program written in Matlab language. The program automatically distinguished cells from relatively dark areas between the cells and performed calculations (in each pixel in the areas containing cells) according to the formula:

$$\text{oxidation}\% = 100\% * \text{Green}/(\text{Green} + \text{Red})$$

where Green and Red are the intensities of the green and red fluorescence signals in a pixel. For ratio imaging, the "oxidation%" was imaged in each pixel as gradations between yellow and red.

Average degree of probe oxidation in a given image was calculated as:

$$(\text{oxidation}\%)_{av} = 100\% * \text{Green}_{av}/(\text{Green}_{av} + \text{Red}_{av}),$$

where Green_{av} and Red_{av} are intensities of the green and red signals, averaged across the areas of the image containing cells.

3. Results

C11-BO is a hydrophobic fluorescent probe, which localize in intracellular membranes and is sensitive to lipid peroxidation. In the first set of experiments, cells were loaded with photosensitizer Pc4 for 3 h, then with C11-BO for 15 min and were illuminated afterwards. Illumination caused oxidation of C11-BO, manifested in an increase of green fluorescence and concomitant decrease of red fluorescence (Fig. 1A–C). After 15 min, a punctate pattern of green fluorescence was observed in many cells, indicating that the oxidative stress was mostly localized to some vesicular organelles (Fig. 1C). In order to quantify the degree of oxidation in the cells, we used ratio imaging, as exemplified in Fig. 1D–E. Only a moderate oxidation of the probe occurred during a relatively short 30 s exposure to light (Fig. 1B and E); a much larger increase in oxidation occurred at 15 min after illumination (Fig. 1C and F). Under these conditions of PDT, most of the cells remained alive during 1 h, whereas after 6 h up to 60% of the cells turned apoptotic (Fig. 1G). Neither significant oxidation of the probe, nor noticeable cell death were observed in "no light" and "no sensitizer" controls.

Fig. 2 shows the average degree of oxidation of C11-BO in the cells subjected to different treatments. Curve 1 corresponds to the experiment presented in Fig. 1. In the cells loaded with Pc4 and then with C11-BO, the degree of probe oxidation before illumination was 3.6% (corresponds to Fig. 1A and D). Illumination of such cells at 0.25 J/cm^2 resulted in an immediate gain in oxidation up to 9.3% (corresponds to Fig. 1B and E). During next 15 min, the extent of oxidation gradually increased up to 30% (corresponds to Fig. 1C and F) and reached a maximum of 36% after 1 h. In order to confirm the "delayed" character of oxidation after PDT and to exclude the possibility of direct photochemical interactions between Pc4 and C11-BO, the probe was loaded into the cells after illumination. In this setup, the same effect of prolonged C11-BO oxidation was observed (curve 2). In untreated cells loaded with C11-BO, the extent of probe oxidation was 2.5% and did not change noticeably during 1 h (curve 5).

In order to find out whether the prolonged oxidation observed is specific for PDT, we performed similar experiments

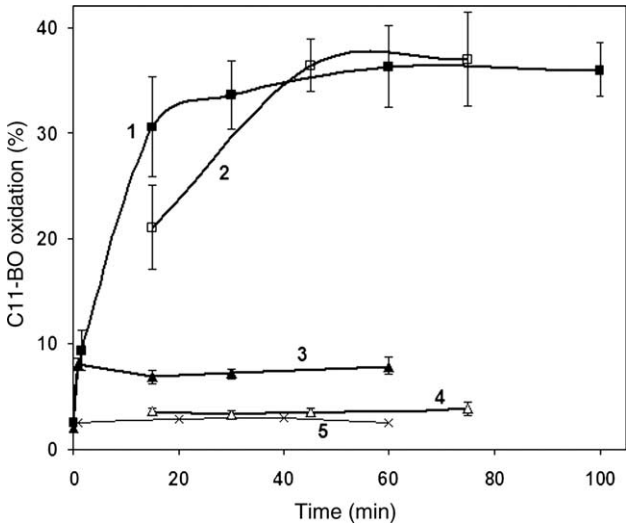

Fig. 2. Oxidation of C11-BO during and after PDT and H_2O_2 exposure. The degree of the probe oxidation was determined by analyzing images of living cells taken with confocal microscope. Average degree of oxidation was calculated for each image, three independent images were analyzed for each experimental condition, data are presented as means ± S.D. Before measuring the degree of oxidation cells were subjected to the following sequential treatments: *1*, loading with Pc4, loading with C11-BO, illumination; *2*, loading with Pc4, illumination, loading with C11-BO; *3*, loading with C11-BO, treatment with H_2O_2; *4*, treatment with H_2O_2, loading with C11-BO; *5*, loading with C11-BO, no treatment. H_2O_2 was incubated with cells for 1 min at 5 mM.

using H_2O_2 as an oxidant. Treatment of C11-BO-loaded cells with different concentrations of H_2O_2 caused progressive oxidation of C11-BO with a rate roughly proportional to the H_2O_2 concentration (not shown). Treatment of cells with 5 mM H_2O_2 for 1 min resulted in 8.4% C11-BO oxidation, comparable to initial gain in oxidation during PDT with Pc4 (9.3%). However, when H_2O_2 treatment was stopped by removing H_2O_2 from the medium, probe oxidation did not go on (Fig. 2, curve 3). In the cells, pre-treated with 5 mM H_2O_2 and then loaded with C11-BO, C11-BO oxidation was barely observed (curve 4). Thus, unlike PDT, H_2O_2 treatment did not cause a prolonged oxidation.

A prolonged oxidation after PDT was observed not only with C11-BO, but also with CM-DCF, another oxidation-sensitive probe, which is frequently used as an overall sensor of oxidative stress in the cells. This probe is homogeneously distributed throughout the cytosol and has a wide spectrum of ROS-sensitivity, in particular it is sensitive to peroxyl and hydroxyl radicals [11]. CM-DCF was added to the Pc4-loaded cells after illumination. When both photosensitizer and light were present, a strong DCF signal was detected (Fig. 3A). There was almost no detectable CM-DCF signal in "no-light" (Fig. 3B) and "no-sensitizer" (not shown) controls. In the post-PDT cells (Fig. 3A), the CM-DCF fluorescence was present throughout the cytosol, yet was most bright in vesicular organelles localized around the nucleus. H_2O_2 treatment of the cells also caused CM-DCF oxidation, but in this case the "vesicular" oxidation pattern was not observed at any H_2O_2 concentration ranging from 25 μM to 5 mM, i.e., the fluorescence was evenly distributed throughout the cytosol (Fig. 3C). The punctate perinuclear oxidation pattern after PDT

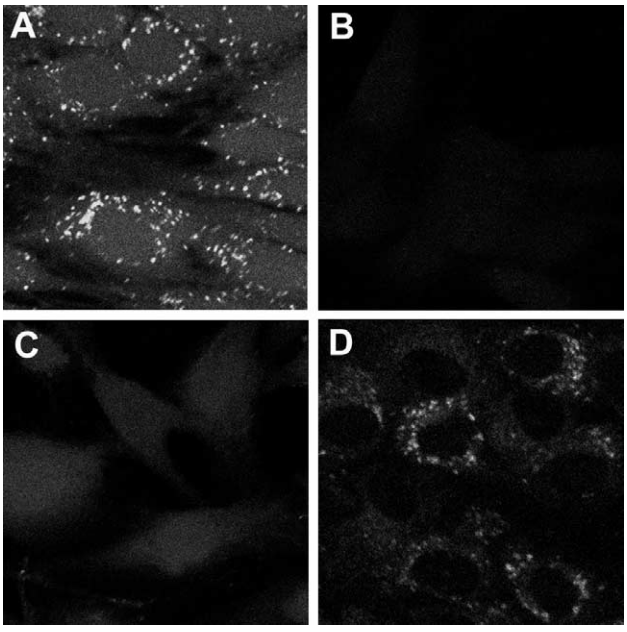

Fig. 3. Oxidation of CM-DCF and BO-C11 after PDT. (A, B) Cells loaded with Pc4 were either illuminated (A) or not (B), loaded with CM-DCF and observed with confocal microscope 2 min after CM-DCF loading. (C) Cells were loaded with CM-DCF and treated with 200 μM H_2O_2 for 15 min. (D) Cells loaded with Pc4 were illuminated, loaded with C11-BO and observed with confocal microscope 15 min after C11-BO loading (only green fluorescence of oxidized C11-BO is shown).

was observed not only with CM-DCF, but also with C11-BO (Fig. 3D). It is also visible in many cells in Fig. 1C.

Fig. 4A shows intracellular distribution of Pc4 after 3 h loading. In agreement with the data published [8], Pc4 accumulates to some extent in mitochondria (Fig. 4B), but it is also present in other organelles, resulting in an almost ubiquitous, although heterogeneous, staining of the whole cell except for the nucleus. The bright punctate CM-DCF staining overlapped, but did not coincide with the Pc4 distribution in the cells (Fig. 4C), being present in a much smaller cellular area than the area containing Pc4. There was no co-localization between mitochondria stained with Mitotracker Red and the ''oxidized'' organelles (Fig. 4D). Lysosomes, visualized with AO in Pc4-loaded cells are shown in Fig. 4E (red fluorescence). Illumination of these cells resulted in disappearance of the lysosomal AO staining (Fig. 4F). This did not happen when the cells loaded with AO only were illuminated under similar conditions (Fig. 4G). After illumination of cells, loaded with Pc4, lysosomes could not be detected anymore with AO added afterwards (Fig. 4G). These data show that, as a result of PDT, lysosomes get damaged. In view of the clear morphological similarity of the ''oxidized'' organelles (Fig. 4C) and lysosomes (Fig. 4E and G), this observation suggests that the ''oxidized'' organelles are most likely damaged lysosomes.

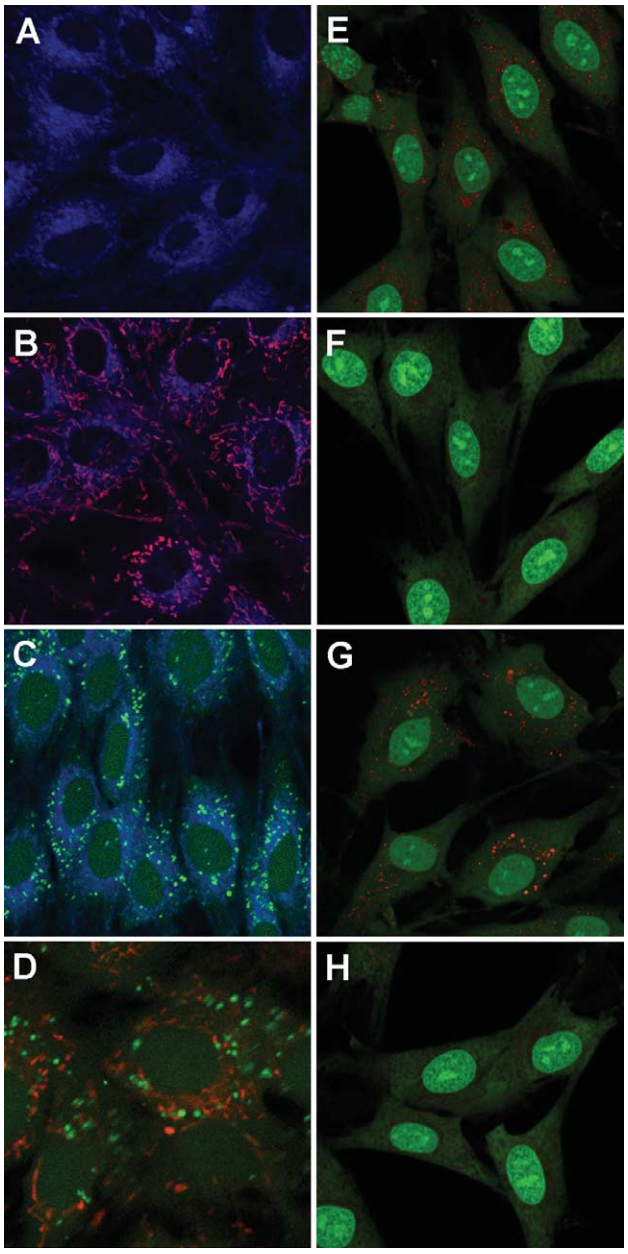

Fig. 4. Visualization of Pc4, cellular organelles and oxidative stress after PDT. (A) Pc4, shown in blue; (B) Pc4 (blue) and mitochondria, stained with Mitotracker Red; (C) Pc4 (blue) and oxidized CM-DCF (green); (D) oxidized CM-DCF (green) and mitochondria (red). (E) AO in Pc4-loaded cells (no illumination); (F) the same after illumination; (G) cells stained with AO (no Pc4) after illumination; (H) cells, loaded with Pc4, illuminated, and stained with AO afterwards.

4. Discussion

Oxidative stress can produce cell injury by multiple pathways [12]. Oxidative reactions in biomembranes are particularly important because they may result in the impairment of lipid–protein interaction, modification and fragmentation of membrane proteins, loss of compartmentalization and membrane integrity, leading thereby to the cell death [12,13]. The major oxidative reaction in biomembranes is lipid peroxidation, which is a free-radical chain reaction capable of propagating in space and time. These reactions are well studied in model systems, although much less is known about lipid peroxidation in living cells.

Most of the methods that measure lipid peroxidation in cells [11–13] are based on the detection of a particular

end product (e.g., malonedialdehyde, 4-hydroxynonenal, isoprostanes, etc.) in cellular extracts. These methods do not provide any information on the subcellular localization of oxidative stress. For the proper understanding of the role of lipid peroxidation in cell physiology it is highly desirable to visualize oxidant activities in living cells on a microscopic level.

Recently, C11-BO was introduced as an oxidation-sensitive probe, suitable for detection of lipid peroxidation in living cells and perfectly compatible with microscopic measurements [9,10]. The most important advantage of this probe is its ratiometric nature: upon oxidation, the excitation and emission spectra of the probe shift to shorter wavelengths. Therefore, the degree of oxidation can be measured quantitatively in each area of a microscopic image by calculating a normalized ratio of green-to-red fluorescence. In this study, we used C11-BO to monitor intracellular localization and propagation of oxidative stress induced by PDT in living cells.

We found that lipid peroxidation in the cells did not happen instantaneously during PDT, but continued to increase during a 15–30 min period. This finding is consistent with the observation by Kessel and Luo [14] that PDT-induced apoptosis can be prevented by antioxidants added to the cells during or immediately after PDT, but not at 10 min after PDT. The authors suggest that PDT generates long-persisting "activated" species, possibly lipid and cholesterol hydroperoxides, capable of apoptosis initiation. Supposedly, we have visualized the intracellular distribution and propagation of these species, assuming that C11-BO is representative for these endogenous lipids.

Lam et al. [15] studied the mitochondrial function in epidermoid carcinoma cells after PDT with Pc4. In line with their observations, we found that Pc4 localizes to some extent to mitochondria, but also to other intracellular organelles. In contrast to Lam et al., who reported that CM-DCF oxidation after PDT co-localizes with mitochondria, we have clearly shown that CM-DCF is predominantly oxidized in vesicular perinuclear organelles, which neither co-localize, nor are morphologically similar to mitochondria. The conclusion by Lam et al. is based on an image where both CM-DCF and Mitotracker signals are very intense and present almost everywhere around the nucleus. This might have lead to a misintepretation of the co-localization. Because of the lack of co-localization of the oxidized probes to mitochondria, we infer that these organelles are not involved in the prolonged PDT-induced oxidative stress, observed in this study.

The observation that the intracellular localization of oxidative stress generated by PDT does not coincide with the intracellular localization of the photosensitizer is intriguing. Both oxidation-sensitive probes used in this study indicated that oxidative stress was localized in a limited number of vesicular perinuclear organelles, whereas the photosensitizer showed a much broader localization in the cytoplasm. The morphological similarity of the "oxidized vesicles" with lysosomes, and the disappearance of AO staining of lysosomes after PDT (Fig. 4E–H) imply that the "oxidized" organelles are most likely damaged lysosomes.

We hypothesize that a specific environment within lysosomes, combining a low pH with high redox-active iron concentration [16,17], could promote lipid peroxidation in these organelles. Lipid hydroperoxides are prominent early products of photoperoxidation that typically arise via singlet oxygen attack. Lipids can undergo an iron-catalyzed one-electron reduction to chain-initiating free radicals, triggering rounds of free radical lipid peroxidation [13] and exacerbating peroxidative damage. Recent data indicate that the major pool of redox-active iron is located within the lysosomes [18], providing possible explanation for the phenomenon observed in this study. Apparent variability in the degree of oxidation between different cells in the same culture dish (Fig. 1C and F) is in line with the observation that the amount of redox-active iron varies between similar cells and between lysosomes in an individual cell [19].

Our data indicate that, unlike PDT, treatment of cells with H_2O_2 does not lead to generation of prolonged oxidative reactions. The reasons for such a difference are not immediately obvious. H_2O_2 itself cannot initiate lipid peroxidation, but, in the presence of iron, it can generate chain-initiating hydroxyl radicals [12], and, thus, could theoretically trigger lipid peroxidation in iron-rich lysosomes. However, we did not observe any preferential lysosomal oxidation at a wide range of H_2O_2 concentrations. A possible explanation is that under our experimental conditions PDT results in a higher local production of chain-initiating radicals in lysosomes, than the H_2O_2 treatment.

In conclusion, the oxidation-sensitive probe C11-BO was instrumental in the visualization of delayed oxidative stress in living cells after PDT. Presumably, this probe will be helpful for characterizing other photosensitizers in respect of intracellular localization and propagation of oxidative stress after PDT.

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