Illumination of the Malaria Parasite Plasmodium falciparum Alters Intracellular pH

IMPLICATIONS FOR LIVE CELL IMAGING*

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Live cell fluorescence microscopy has been widely used to study physiological processes in the human malarial parasite Plasmodium falciparum, including pH homeostasis, Ca2+ signaling and protein targeting. However, the reproducibility of the data is often poor. Controversial statements exist regarding cytosolic and vacuolar baseline pH, as well as regarding the subcellular localization of some of the fluorochromes used. When trying to reproduce published baseline values, we observed an unexpected light sensitivity of P. falciparum, which manifests itself in the form of a strong cytoplasmic acidification. Even short exposure times with moderate to low light intensities caused the parasite cytosol to acidify. We show that this effect arises from the selective disruption of the parasite’s acidic food vacuole, brought about by lipid peroxidation initiated by light-induced generation of hydroxyl radicals. Our data suggest that heme serves as a photosensitizer in this process. Our findings have major implications for the use of live cell microscopy in P. falciparum and add a cautionary note to previous studies where live cell fluorometry has been used to determine physiological parameters in P. falciparum.

Progress in understanding the physiology of the human malarial parasite Plasmodium falciparum has been slow, despite the fact that P. falciparum is a major health problem, killing millions of humans every year (1). As a consequence, only a limited number of potential drug targets have been identified in recent years. One reason for this is the obligatory intracellular life style of the parasite, which has complicated access to biochemical and physiological pathways.

A powerful method to investigate physiological processes of intact cells, under non-disruptive conditions, is live cell fluorescence microscopy. This technique has recently been applied to the intraerythrocytic stages of P. falciparum for measuring intracellular pH, ion concentrations, Ca2+ signaling, and protein targeting (2–7). The main advantage of live cell fluorescence microscopy is that it allows for the spatial separation of signals originating from the parasite and from the surrounding infected erythrocyte. Even subcellular compartments, such as the parasite’s acidic food vacuole, can be visualized in situ using this method (5). However, a number of concerns have recently been raised about using single cell fluorescence microscopy in P. falciparum (8).

A major disadvantage is the variability between cells, requiring a relatively large number of determinations to obtain statistically significant measurements. Reproducibility between different laboratories seems to be low as well when data obtained from single cell measurements are compared. For example, although some studies have found differences in the parasite’s cytosolic pH between chloroquine-sensitive and chloroquine-resistant P. falciparum strains, other studies could not verify this observation (7, 9–12). Another example represents the use of acridine orange (AO)1 in the measurement of the parasite’s food vacuolar pH (5, 13–15). Although AO is an acidotropic compound and expected to accumulate in the parasite’s acidic food vacuole, subsequent studies have challenged this concept by presenting data that show exclusive staining of the parasite’s cytosol with AO, while the parasite’s food vacuole remained unstained (14). Thus, the intracellular distribution of AO within the parasite is still uncertain.

Where do these discrepancies come from? In the case of AO, interpretation of the data is complicated by the fact that AO has very different fluorescence properties depending on its concentration and its interaction with cellular macromolecules, e.g. DNA and RNA (16–18). When excited at 490 nm, monomeric AO emits green light of ~530 nm. These spectral properties remain unchanged when AO binds to double-stranded DNA, thus staining nuclei green. At higher concentrations AO forms aggregates, causing a major shift in the emission spectrum toward red light of around 655 nm (18). AO also shows red fluorescence when bound to single-stranded RNA. Because AO is a lipophilic weak base, it is readily membrane-permeable and can accumulate in acidic compartments, where the protonated molecule forms red fluorescing aggregates. Therefore, acidic compartments stain red with AO, a phenomenon often used to identify lysosomes or other acidic organelles (18).

Due to the varying spectral properties of AO, the right choice of filters allowing the green from the red fluorescence to be separated is essential to quantify the emission signal and obtain accurate data on pH and subcellular localization. Furthermore, because aggregation is strongly dependent on a number of parameters, including pH gradients, AO concentration, temperature, and

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1 The abbreviations used are: AO, acridine orange; CM-H2DCFDA, (5 and 6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate; BODIPY FL C10, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid; SNARF, (5 and 6)-carboxyl SNARF-1-AM; BCECF, 2’,7’-bis-(2-carboxyethyl)-5 and 6-carboxyfluorescein acetoxymethyl ester; FACS, fluorescence-activated cell sorting; GFP, green fluorescence protein.
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EXPERIMENTAL PROCEDURES

P. falciparum Culture—In vitro culture of the P. falciparum strain HB3 was performed as described previously (19).

Dye Loading—Unless stated otherwise, P. falciparum-infected erythrocytes of mixed stages were washed twice with Ringer’s solution and settled onto poly-l-lysine-coated coverslips in a micro-perfusion chamber as previously described (7, 20). Unbound parasites were washed away by perfusion with Ringer’s solution supplemented with the fluorescence dyes at the following concentrations: acridine orange, 1–5 μM; Lysosensor Blue DND 192, 1 μM; Hoechst 33342, 1 μM. In other experiments, infected erythrocytes were pre-loaded with the dye re-suspended in Ringer’s solution for 10 min at 37 °C. The following dye concentrations were used: (5 and 6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), 10 μM; 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-hexadecanoic acid (BODIPY FL C12), 10 μM; (5 and 6)-carboxyl SNARF-1-AM (SNARF), 10 μM; 2’,7’-bis(2-carboxyethyl)-5 (and 6)-carboxyfluorescein acetoxymethyl ester (BCECF), 10 μM. The remaining dye was washed out by perfusion with Ringer’s solution. Acridine orange was purchased from Sigma; all other dyes were obtained from Molecular Probes. The internal pH calibration of the SNARF fluorescence ratios was performed using the high K+/nigericin method as previously described (7, 20).

Single Cell Imaging—Epifluorescence images of acridine orange fluorescence were obtained with an inverted Zeiss Axiovert 100 microscope. Mono-chromatic excitation light was generated by a xenon lamp coupled to a Polychrome II monochromator (Till Photonics, Germany) set to the wavelength indicated for the different experiments. Between exposures the wavelength was rapidly switched to 680 nm as a shutter wavelength. The excitation light was reflected onto the sample with a 510-nm dichroic mirror (Delta Lights and Optics, Denmark) and focused by a Zeiss Fluor 100/1.3 oil lens. The emitted light passing back through the dichroic mirror was filtered by a band pass filter D 630/60 (AHF Analysetechnik, Germany) and imaged using a cooled charge-coupled device camera (Princeton Instruments). Data acquisition and analysis were performed using Metafluor (Universal Imaging Corp.).

Confocal scanning fluorescence microscopy was performed using a Zeiss LSM510 (Carl Zeiss, Germany) equipped with UV and visible laser lines. The laser lines and filter settings used were as indicated in the figure legends. For optimized pH measurements we used the following settings: 10 μM SNARF, loaded at 37 °C for 20 min; excitation of 543 nm with 3–5% transmission, 63-fold lens with a 5-fold zoom, 256 × 256 pixels with a 1.76-μs pixel time, and no pixel averaging.

Irradiation Dose Response—P. falciparum-infected erythrocytes were purified using a strong magnet (VarioMACS, Miltenyi Biotec, Germany) and 6)-carboxyl SNARF-1-AM (SNARF), 10 μM; (5 and 6)-carboxyfluorescein acetoxymethyl ester (BCECF), 10 μM. The remaining dye was washed out by perfusion with Ringer’s solution. Acridine orange was purchased from Sigma; all other dyes were obtained from Molecular Probes. The internal pH calibration of the SNARF fluorescence ratios was performed using the high K+/nigericin method as previously described (7, 20).

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Acidification of the parasite by illumination. a, confocal images of infected erythrocytes loaded with carboxyl SNARF (5 μM) and with 1 μM Lysosensor Blue DND 192 added to stain the vacuole. While SNARF preferentially stained the parasite cytosol (red channel), Lysosensor Blue accumulated into the acidic food vacuole (green channel). SNARF was excited at 543 nm (19% transmission), and the emission was recorded for the two pH-sensitive wavelength bands from 560 to 610 nm and above 650 nm to calculate the signal ratio. Lysosensor Blue was excited at 364 nm (2% transmission) in the line multitrack mode, and emission was recorded from 385 to 470 nm. Scale bars, 2 μm. b, time course of the Lysosensor Blue fluorescence imaged at 5-s intervals. The intensities are expressed as the ratio of the average vacuolar intensity divided by the average cytosolic intensities. c, time course of the cytosolic pH from the same recording calculated from the SNARF ratios after an internal calibration was performed at pH 7.4, 7.0, and 7.8 as described previously (7, 20). This recording is representative of four similar measurements.

The light-induced acidification of P. falciparum was characterized using two ratiometric probes: SNARF and Lysosensor Blue. SNARF is a fluorescent dye that changes its emission properties in response to changes in pH, while Lysosensor Blue is a weak base that accumulates in acidic compartments and is not affected by changes in temperature. In this study, the acidification process was monitored using a confocal laser-scanning microscope, which allowed for the visualization of the pH changes within the parasite's vacuole and cytoplasm.

RESULTS

AO Distribution Is Dynamic in P. falciparum—To evaluate a method recently introduced for measuring the pH of the acidic food vacuole in P. falciparum using AO (5), we initially characterized the subcellular distribution of AO fluorescence in living P. falciparum-infected erythrocytes. We observed a complex fluorescence pattern (Fig. 1). The highest intensity from the red emission band (>650 nm) was observed in a defined area covering the dark hemozoin crystal. This staining pattern is consistent with AO being trapped in the acidic food vacuole upon protonation. The green emission band (505–550 nm) co-localized with the fluorescence of Hoechst 33342, a DNA marker, which indicates association of AO with nuclear DNA.

We also observed a significant number of parasites with an inverted staining pattern for the red fluorescence band, i.e. instead of the vacuole, the cytoplasm was stained. In comparison, the green fluorescence pattern remained unchanged. Examinations of single cells revealed that the redistribution of the red AO fluorescence from a vacuolar to a cytoplasmic localization is a dynamic process that occurs during continuous illumination. Although initially the bright red AO fluorescence exclusively co-localized with the parasite’s acidic food vacuole (Fig. 2a), a redistribution toward the cytosol was observed after 400 s of illumination, corresponding to 80 exposures with 0.1% transmission of the 488 nm argon laser line (Fig. 2b). Quantification of the fluorescence signals revealed that the decline in vacuolar red AO fluorescence is linear with time and occurred, after a lag phase of ~150 s, concomitantly with an increase in the cytosolic red fluorescence (Fig. 2c). The decline in fluorescence intensity was not due to bleaching of the dye, because the green fluorescence intensities showed a marked increase throughout the measurement. Inversion of the red AO fluorescence signal was independent of the light source used and was observed using a laser scanning microscope as well as a conventional epifluorescence microscope with a 75-watt xenon short-arc lamp for illumination, exciting at 480 nm with a 99.9% absorption pre-filter (Fig. 2d). Measurements were conducted at 25 °C in an open bath chamber or at 37 °C in a closed recording chamber continually perfused with RPMI medium, therefore ruling out the possibility that changes in temperature or different buffer composition caused inversion of the red AO fluorescence pattern. With both types of imaging, we did not observe the crescent shaped AO staining pattern previously described (5), unless the dye was cytosolic (Fig. 2h).

Redistribution of AO Fluorescence Results from a Cytosolic Acidification—Because the subcellular distribution of AO is pH-dependent, we investigated whether the inversion of the red AO fluorescence pattern was due to changes in vacuolar and cytosolic pH. We incubated P. falciparum-infected erythrocytes with the lysosomal marker Lysosensor Blue DND 192 and the ratiometric pH sensor SNARF. Like AO, Lysosensor Blue is a weak base that accumulates in acidic compartments but does not interact with DNA or RNA. The fluorescence signals emitted from Lysosensor Blue and SNARF were monitored using a confocal laser-scanning microscope. Initially, Lysosensor Blue preferentially stained the food vacuole, whereas SNARF showed a strong staining throughout the par-
Light-induced Acidification in *P. falciparum*

In the case of Lysosensor Blue, the subcellular distribution changed upon prolonged illumination in a manner similar to that observed for AO, i.e., a drastic decrease in vacuolar staining and an increase in cytosolic staining (Fig. 3b). The subcellular redistribution of the Lysosensor Blue fluorescence signal coincided with a marked decrease in the cytoplasmic pH of at least 0.45 pH unit, as observed by simultaneous pH measurements using the ratiometric dye SNARF (Fig. 3c). In all cases in which we observed light-induced cytoplasmic acidification, the observed pH reached the lower end of the SNARF calibration curve (pH < 7.0), suggesting that the actual pH might have dropped even further. This was confirmed using the fluorophore calcein, which is only fluorescent above pH 6.0 (23). Upon illumination of the parasite, the calcein fluorescence vanished, suggesting a drop in cytosolic pH below 6.0 (data not shown).

These light-induced changes in the parasite’s cytoplasmic pH were observed independent of the pH indicator used. The pH-sensitive fluorescent dye BCECF, which has been previously used to determine the parasite’s cytoplasmic pH (6, 7, 20), also monitored changes within the parasite’s cytosolic pH during illumination (Fig. 4). Once the laser started scanning the individual cells, the subsequent scan of the same erythrocytes. Note also the almost complete loss of BCECF signal from the parasite cytosol (Fig. 4c), from which the parasite recovered only slowly. A stable cytosolic pH could only be measured under optimized conditions with a minimal amount of illumination (Fig. 5b) (543-nm excitation, low transmission, few scan points per cell, minimal pixel time, no pixel averaging).

**Light-induced Acidification Is Dose- and Wavelength-dependent**—We next set out to determine the effect of exposure time and wavelength on the light-induced cytoplasmic acidification in *P. falciparum*, using the intracellular redistribution of AO as a marker. Cells were loaded with AO and exposed to different light pulses ranging from 20 ms to 1000 ms, while keeping the wavelength of the pulse constant at 480 nm and the transmission at 0.1% (Fig. 6a). We observed a reverse correlation between the length of the light pulse and the ratio of the red AO fluorescence determined in the vacuole versus that of the cytoplasm. Thus, the longer the light pulse, the faster the subcellular redistribution of AO occurred. It was noticeable that even minimal exposure times of 20 ms led to an inversion of the AO staining with a half-life time of about 200 s (Fig. 6b).

To quantify the dose of irradiation required to induce changes in cytoplasmic pH, infected erythrocytes were loaded with SNARF and exposed at a constant distance to the beam of a 20-watt halogen lamp for various lengths of time ranging from 5 to 1200 s. The irradiation emitted during each exposure was quantified using a photodiode connected to a calibrated quantum sensor. Changes in cytosolic pH were then ratiometrically determined using a FACSCalibur (22). A sharp decrease in cytoplasmic pH of at least 0.4 pH unit, from a resting pH of 7.3 ± 0.05 (n = 6) to below 6.9, was observed upon illumination. A graphic analysis of the cytosolic pH as a function of irradiation revealed a sigmoidal dose-response curve, with a half-maximal drop in cytoplasmic pH at 1700 ± 400 mmol of photons/m² (Fig. 7a). However, the first effects on cytoplasmic pH were already observed with an irradiation of 120 mmol of...
photons/m². A similar sigmoidal relationship between irradiation and changes in cytoplasmic pH was also obtained using BCECF as the pH indicator (Fig. 7b). Given that BCECF requires excitation at 460 and 488 nm for ratiometric determinations, conditions that the FACSCalibur used in this study does not support, the BCECF emission fluorescence signal recorded in the FL-1H channel of the FACSCalibur could not be converted into pH values. Despite these limitations, the changes in BCECF fluorescence observed upon irradiation with light provides a qualitative measure for changes in cytoplasmic pH. Analysis of the forward and side scattering revealed no changes in the cell volume of infected erythrocytes (data not shown), indicating that the integrity of the erythrocyte remained intact during the entire procedure.

To determine the wavelength dependence of the light-induced changes in P. falciparum, we loaded infected erythrocytes with AO and exposed these to two different wavelengths: one of 480 nm to measure AO fluorescence (50 ms), and a second variable wavelength of either 350, 400, or 450 nm, respectively, for 200 ms (Fig. 6c). Changes in AO fluorescence were monitored by confocal laser scanning microscopy. The transmission efficiencies of the optics at the different wavelengths were comparable (±10%), as determined using a photodiode in the sample holder. We observed the slowest response in terms of AO redistribution at 350 nm with a time of half-maximal change of 140 s, whereas at both 400 and 450 nm the half-times were about 30% shorter. This implies that the inversion of the AO staining is more sensitive to visible light than to UV light.

Light-induced Cytosolic Acidification Results from the Lysis of the Food Vacuole—To test whether the light-induced pH
changes and the subsequent dye redistribution might be caused by lysis of the parasite acidic food vacuole, we examined the autofluorescence from this compartment under continued light exposure. Illuminated at 488 nm (30% transmission), the food vacuole emits a weak autofluorescence together with some reflection from the hemozoin crystal (Fig. 8a). This autofluorescence filled out the entire vacuole and co-localized with the vacuolar dye Lysosensor Blue DND 192 (Fig. 8a). Under these conditions, no autofluorescence was observed from the parasite cytoplasm. However, upon repeated illumination, the Lysosensor Blue started to redistribute and, concomitantly, the autofluorescence spread from the vacuole until it filled the entire cytosol (Fig. 8b). A similar change in the autofluorescence pattern could also be observed in the absence of Lysosensor Blue under otherwise identical conditions, ruling out a bleed-through between recording channels used to monitor the different fluorescence signals emitted (data not shown). These data suggest that the parasite’s acidic food vacuolar membrane loses its integrity during repeated illumination.

**Lysis of Food Vacuole Correlates with Light-induced Production of Radicals and Lipid Peroxidation**—It has been shown in other systems that membrane damage can result from peroxidation of polyunsaturated fatty acids (24, 25). A highly effective substance to initiate lipid peroxidation, a self-perpetuating chain reaction, is the hydroxyl radical (24, 25). To determine whether light generates hydroxyl radicals in *P. falciparum* we took advantage of a substance, called CM-H$_2$DCFDA, which emits fluorescence when associated with a hydroxyl radical (26). Thus, the fluorescence intensity emitted by CM-H$_2$DCFDA directly correlates with the amount of hydroxyl radicals generated in the cell (26). *P. falciparum*-infected erythrocytes were loaded with CM-H$_2$DCFDA and exposed to different intensities of the 488-nm argon laser line. Changes in CM-H$_2$DCFDA fluorescence were recorded over time using a confocal laser scanning microscope (Fig. 9). At 0.1% light transmission, CM-H$_2$DCFDA fluorescence increased only slightly during the recording time of 300 s. At higher transmission intensities, however, a marked increase in CM-H$_2$DCFDA fluorescence occurred, indicating an increased generation of hydroxyl radicals in the parasite’s cytoplasm (Fig. 9a). The kinetics of CM-H$_2$DCFDA fluorescence, and hence of hydroxyl radical generation, directly correlated with light intensity (Fig. 9b). With more light, more hydroxyl radicals were produced. The decline in fluorescence observed for 5 and 2% light transmission, once maximal fluorescence values were reached, is due to the lysis of the food vacuole and the subsequent dilution of the dye within the cell.

We next set out to investigate whether the light-induced generation of hydroxyl radicals initiates lipid peroxidation. To this end, *P. falciparum*-infected erythrocytes were loaded with BODIPY FL C$_{16}$, a fluorochrome that integrates into membrane bilayers. Oxidation of the polyunsaturated portion of this end, *P. falciparum*-infected erythrocytes were loaded with BODIPY FL C$_{16}$, a fluorochrome that integrates into membrane bilayers. Oxidation of the polyunsaturated portion of the dye within the cell.

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Our data demonstrate that the malarial parasite *P. falciparum* is extremely light-sensitive. Even moderate to low exposure to light causes irreversible acidification of the parasite cytosol of as much as 1 pH unit. As a consequence, the cytoplasmic pH decreases from its resting value of 7.3 to 6.0 and below. This phenomenon is strongly dependent on the dose of light irradiation, with a half-maximal effect observed at an irradiation dose of 1700 mmol of photons/m². This corresponds to 7 min of illumination with a 20-watt halogen lamp. However, the first significant decrease in cytoplasmic pH is already noticeable after short light exposures of 30–60 s.

Cytoplasmic acidification coincides with leakage of acido-tropic dyes from the parasite food vacuole into the cytoplasm. Similarly, autofluorescence initially contained within the food vacuole spreads into the cytosol upon light exposure of the parasite. On the basis of our data we conclude that the parasite’s food vacuolar membrane is highly susceptible to photo-induced damage and begins to disrupt upon light exposure. The subsequent release of the vacuolar acid load would explain the observed cytoplasmic acidification.

All living cells, to some degree, are susceptible toward photoinduced damage. Molecular absorption of photons by organic molecules results in energy-rich states, which can lead to the generation of free radicals causing uncontrolled reactions in the cell. Energy-rich UV light is particularly damaging, whereas light of longer wavelength is less so. However, in the case of *P. falciparum*, it appears that UV light is less effective in causing damage of the food vacuolar membrane than visible blue light. This is surprising, because polyunsaturated fatty acids, which are most sensitive of irradiation-induced lipid peroxidation, absorb in the short UV band. Thus, the lipid peroxidation observed upon continuous irradiation of the parasite must be initiated by the intervention of some other molecule. As our data suggest, this molecule is most likely localized in the parasite’s cytosol and absorbs visible light between 400 and 450 nm. This observation, together with the fact that the observed extreme light susceptibility of the parasite’s food vacuole is an unexpected result not found for acidic vacuoles of other organisms, including those of plants, would suggest that a parasite-specific factor serves as a photosensitizer.

Obvious candidates are flavins and heme, which absorb light of 400–450 nm. For quantitative reasons and because the presence of free and nonspecifically bound heme is characteristic for *P. falciparum*-infected erythrocytes, we will focus on this molecule. Heme is released in large amounts by the parasite as a byproduct of hemoglobin degradation. Although heme accumulates in the parasite’s food vacuole, where it crystallizes

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**FIG. 9.** Effect of light on the production of hydroxyl radicals in *P. falciparum*. Infected erythrocytes were loaded with the hydroxyl radical sensor CM-H$_2$DCFDA, and the time course of changes in the fluorescence signal were monitored at different light intensities (0.1%, 1%, 3%, and 5% transmission). Fluorescence signals were recorded using a confocal laser scanning microscope (excitation at 488 nm, emission above 505 nm). a, the confocal images show the fluorescence after 24 s of light exposure with the transmission indicated. Bar, 2 μm. b, temporal changes in fluorescence as a function of light transmission. The decline in fluorescence observed for 5% and 2% light transmission, once maximal fluorescence values were reached, is due to the lysis of the food vacuole and the subsequent dilution of the dye within the cell. This recording is representative of four or more similar measurements.

**FIG. 10.** Effect of light on lipid peroxidation in *P. falciparum*. Infected erythrocytes were loaded with BODIPY FL C$_{16}$, a ratiometric sensor of lipid peroxidation. Oxidation of the polyunsaturated portion of the dye results in a shift of the fluorescence emission peak from 590 to 510 nm. a, confocal images showing dye loading and fluorescence at 510 nm (green) and 590 nm (red). Bar, 2 μm. b, temporal changes in fluorescence ratios as a function of light transmission given in percent. This recording is representative of three or more similar measurements.
to hemozoin (32), a significant portion diffuses into the cytoplasm, where it is subject to glutathione-dependent degradation (33). In addition, heme is a prosthetic group for several parasite enzymes.

The fact that we observed a rapid increase in the production of hydroxyl radicals during irradiation also points toward heme as a key player in photosensitization. Heme contains iron, a transition metal ion, which when present as ferrous iron (Fe\(^{2+}\)) catalyzes the Fenton reaction, thereby yielding hydroxyl radicals (OH\(^{-}\)), as follows,

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{-} + \text{OH}^{-}
\]  
(1)

Ferric iron can then react with superoxide ion (O\(_2^\cdot\)) to recycle ferrous iron. The parasite generates both hydrogen superoxide (H\(_2\)O\(_2\)) and O\(_2^\cdot\) in substantial quantities as a result of hemoglobin degradation (34). In other systems the intracellular concentration of H\(_2\)O\(_2\) and O\(_2^\cdot\) are tightly controlled by the enzymatic activities of the superoxide dismutase, catalase, and peroxidase. Whereas the parasite encodes its own superoxide dismutase (35), the catalytic activity associated with \(P. falciparum\)-infected erythrocytes originates from the host erythrocyte (36). Although the erythrocyte catalase can be detected in the parasite’s food vacuole, it is not yet clear whether it maintains its activity in the parasite (36). Instead of a catalase the parasite appears to use a glutathione peroxidase to reduce H\(_2\)O\(_2\) (37, 38).

These natural defense mechanisms against reactive oxygen species are apparently out-competed when parasites are exposed to light, as indicated by the substantial amounts of hydroxyl radicals generated. The hydroxyl radical is a highly reactive oxygen species, which may attack all classes of biomolecules, but lipids are the most likely targets (24, 25). Particularly susceptible are polyunsaturated fatty acids, because reactions with hydroxyl radicals proceed as a self-perpetuating chain reaction (24, 25).

Lipid peroxidation dramatically alters membrane fluidity and permeability and may eventually induce widespread membrane damage, including disruption (24, 25). Our observation that the parasite’s food vacuolar membrane is more susceptible to lipid peroxidation than the plasma membranes of the parasite or host erythrocyte is a reflection of the different contents of polyunsaturated fatty acids, which is usually higher in vacuolar membranes (39). This explains why the food vacuole under conditions where the parasite plasma membrane and the erythrocyte plasma membrane remain intact and are still able to contain fluorescent dyes (Figs. 2 and 3).

Our findings have major implications for all studies using live cell imaging. Because light easily induces lipid peroxidation in \(P. falciparum\), extreme care has to be taken to define conditions that avoid or at least minimize photolysis of the food vacular membrane. In previous studies, it is possible that some of the data generated using live cell imaging have been affected by photo-induced damage to the cell. For example, the controversy surrounding the subcellular localization of the AO fluorescence in \(P. falciparum\) (5, 14) can be resolved when our findings on light sensitivity are taken into account. Because AO itself is a photosensitizer of oxidative reactions (40), the light sensitivity of the parasite is compound-mediated. In the presence of AO, light leads to lipid peroxidation and leakage of the food vacular membrane. The subsequent release of the vacular acid load into the cytoplasm causes a redistribution of the dye and an inversion of the red AO fluorescence staining pattern from a vacular to a cytoplasmic localization. Thus, the red AO fluorescence signal is only observed in the parasite’s cytoplasm upon lysis of the food vacuole and as such represents an artificial condition.

Therefore, studies using AO to determine the food vacular pH of \(P. falciparum\) have to be considered with caution. The reported crescent-shape staining pattern allegedly attributed to the food vacuole could only be observed when AO was in the cytosol after damage of the food vacuolar membrane. Moreover, the reported pH values below 6.0 for the food vacuole (5) are most likely a reflection of the pH of the cytosol after lysis of the food vacuole. That such a strong cytosolic acidification may indeed be achieved upon lysis of the food vacuole is supported by our data showing that calcein looses its fluorescence during continued illumination. This effect is only observed at pH values below 6.0 (23).

Photo-induced damage and subsequent leakage of the acidic food vacuole may also have influenced previous measurements of the parasite’s cytosolic pH. This could explain the wide range of cytoplasmic baseline pH values reported for \(P. falciparum\) (6, 7, 12, 41). Other studies affected in this way may be those using green fluorescence fusion proteins (GFP) as reporters for transport processes in \(P. falciparum\). Because GFP fluorescence is pH-sensitive (42), protonophores such as nigericin (in high K\(^+\) buffer) should be added to the medium to avoid misinterpretation of GFP localization in the cell due to photo-induced lysis of the food vacuole and acidification of the parasite’s cytoplasm.

The inherent light sensitivity of \(P. falciparum\) certainly complicates live cell microscopy of the parasite. However, artifacts caused by photo-induced damage can be avoided when appropriate conditions are chosen allowing, for example, stable pH determinations to be made (Fig. 5b). It is unclear whether light sensitivity plays a physiological function in \(P. falciparum\) biology. Parasites are potentially exposed to light when circulating through the small blood vessels of the skin. Whether this can act as a functional signal (day-to-night synchronization, gametogenesis) still remains to be seen.

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