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Singlet Oxygen Reacts with 2′,7′-Dichlorodihydrofluorescein and Contributes to the Formation of 2′,7′-Dichlorofluorescein

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ABSTRACT

There are controversial reports in the literature concerning the reactivity of singlet oxygen ($^1O_2$) with the redox probe 2′,7′-dichlorodihydrofluorescein (DCFH). By carefully preparing solutions in which $^1O_2$ is quantitatively generated in the presence of DCFH, we were able to show that the formation rate of the fluorescent molecule derived from DCFH oxidation, which is 2′,7′-dichlorofluorescein (DCF), increases in D_2O and decreases in sodium azide, proving the direct role of $^1O_2$ in this process. We have also prepared solutions in which either $^1O_2$ or dication ($MB^{\bullet 2+}$) and semi-reduced ($MB^{\bullet}$) radicals of the sensitizer and subsequently super-oxide radical ($O_2^{\bullet -}$) are generated. The absence of any effect of SOD and catalase ruled out the DCFH oxidation by $O_2^{\bullet -}$, indicating that both $^1O_2$ and $MB^{\bullet 2+}$ react with DCFH. Although the formation of DCF was 1 order of magnitude larger in the presence of $MB^{\bullet 2+}$ than in the presence of $^1O_2$, considering the rate of spontaneous decays of these species in aqueous solution, we were able to conclude that the reactivity of $^1O_2$ with DCFH is actually larger than that of $MB^{\bullet 2+}$. We conclude that DCFH can continue to be used as a probe to monitor general redox misbalance induced in biologic systems by oxidizing radicals and $^1O_2$.

INTRODUCTION

The diverse roles of reactive oxygen and nitrogen species in biology and medicine translate into increased interest in measuring and understanding redox balance in cells (1). Although extremely used in biochemistry and in cell biology to sense oxidative stress, the reactivity of 2′,7′-dichlorodihydrofluorescein (DCFH) against several reactive oxygen and nitrogen species (ROS and NOS, respectively) is still not conclusively defined (2–5).

DCFH is a nonfluorescent compound that after oxidation gives rise to 2′,7′-dichlorofluorescein (DCF), a strong fluorescent molecule. Its diacetate form, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), has been widely applied in cell studies, because it easily penetrates membranes and it is enzymatically hydrolyzed by intracellular esterases to form the nonpermeable DCFH (2–7). It was initially thought to be useful as a specific indicator for intracellular H_2O_2 (2–5). However, it has been already demonstrated that DCFH is not oxidized directly by H_2O_2, instead it reacts with other ROS and NOS generated by intracellular peroxidases (6). Some authors have suggested that it is also oxidized by singlet oxygen ($^1O_2$), although this has never been shown experimentally (2–5,7,8). In fact, Bilski *et al*. provided evidence that $^1O_2$ does not directly react with DCFH (9). Considering that DCFH is a widespread probe for estimating oxidative stress in cells, it is important to know whether or not it reacts with $^1O_2$.

Generating ROS species specifically is always a challenge. However, we have shown that methylene blue (MB) under photoexcitation can be directed to form either $^1O_2$ or semi-reduced ($MB^{\bullet}$) and dication ($MB^{\bullet 2+}$) radicals and subsequently anion radical superoxide ($O_2^{\bullet -}$) (10–13). Monomer and dimer species of MB have different absorption spectra and the dimerization equilibrium is modulated by the presence of anionic interfaces including sodium dodecyl sulfate (SDS) micelles (10,11), other membrane mimetic systems (12) and mitochondria (13). Depending on the MB local interfacial concentration either monomers or dimers are favored, shifting the triplet deactivation mechanism from energy transfer and formation of $^1O_2$ to electron transfer and formation of $MB^{\bullet}$ and $MB^{\bullet 2+}$ radicals. $O_2^{\bullet -}$ is subsequently formed by the reaction of molecular oxygen with $MB^{\bullet}$ (Scheme 1) (10,12).

There has been interest in designing inexpensive photodynamic therapy protocols (PDT) (14–16). DCFH (in its diacetate form) offers an interesting option to investigate the general oxidative stress induced in cells and tissues by photosensitizers that are generally excellent sources of $^1O_2$ (14–20). However, it is imperative to know whether or not DCFH reacts with $^1O_2$. By using known controls of the photochemical events related with $^1O_2$ and the controlled photochemistry of MB in the presence of anionic interfaces, we were able to set up experimental protocols to elucidate whether or not DCFH reacts with $^1O_2$ and with other radicals derived from MB photoexcitation.

MATERIALS AND METHODS

Sodium azide, SDS, deuterium oxide (D_2O), superoxide dismutase CuZnSOD (SOD) and catalase were purchased from Sigma and used as received. Methylene blue (MB-Aldrich) was double recrystallized from ethanol. Solutions were prepared in Milli-Q water (pH $\sim$ 6.5).

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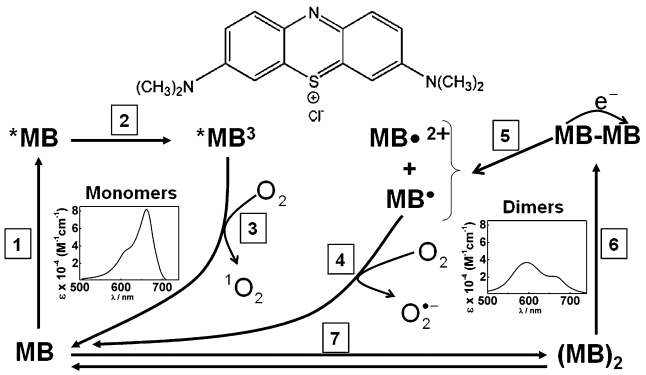

Scheme 1. Methylene blue (MB) photochemical reaction routes where MB, MB*, ^{3}MB* are MB ground state, singlet and triplet excited states, respectively, $MB^{\bullet}$ and $MB^{\bullet 2+}$ are MB semi-reduced and dication radicals, respectively. Numeric pathways correspond to: (1) light absorption of monomeric MB species (λ_{max} = 664 nm), (2) intersystem crossing, (3) reaction of ^{3}MB* with molecular oxygen forming singlet oxygen ($^1O_2$), (4) oxidation of $MB^{\bullet}$ by molecular oxygen returning the ground state dye and forming anion superoxide radical ($O_2^{\bullet -}$), (5) redox suppression of (^{3}MB- - - - -MB)* after exciting ground state dimers, (6) light absorption of dimeric MB species (λ_{max} = ~590 nm), (7) ground state dimerization. The relative position of the species presented in this scheme does not represent their actual energy levels. Modified from Junqueira *et al.* (10).

DCFH (D6883; SIGMA) was obtained by hydrolysis of its diacetate form (DCFH-DA, 2′, 7′-dichlorodihydrofluorescein diacetate). Briefly, an appropriate aliquot from a DCFH-DA stock solution (200 μM) was added to an aliquot of NaOH (0.5 M) in a fluorescence quartz cuvette in the dark and kept on ice for 30 min and used the same day. The hydrolyzed solution was then neutralized with an appropriate volume of sulfuric acid to pH 7.2. The pH value was measured by means of a glass electrode.

Aqueous solutions composed of either monomers or dimers of MB were prepared by mixing MB into 30 or 1 mM SDS, respectively (10,12). MB molar concentrations and aggregation state were checked by absorption spectra measurements (Shimadzu UV–VIS 2400-PC spectrophotometer).

DCF formation. DCFH and MB-containing samples were irradiated by a CW 664 nm diode laser, 50 mW (Morgotron, Brazil), in a stirred quartz cuvette (10 mm optical path length) that was assembled in the cuvette-holder of a SPEX FLUOROG spectrofluorimeter. Fluorescence spectra were obtained in the right-angle mode, using the excitation wavelength of 498 nm. All measurements were made just after a specific irradiation procedure, at room temperature. Spectral data were further manipulated with a 386 GRAMS (Galatic, Inc.) and/or Origin (Microcal) softwares.

$^1O_2$ phosphorescence NIR emission produced by photo-excitation of MB was obtained as previously described (12,13). Briefly, a Nd:YAG (10 mJ/pulse; 5 ns/pulse) Surelite III laser (Continuum) excites MB species at 532 nm, into a fluorimeter (Edinburgh Analytical Instruments) that is connected to a near-infrared-PMT (R5509 from Hamamatsu Co.). The phosphorescence emission of $^1O_2$ was recorded in the 1200–1310 nm spectral region, and the $^1O_2$ lifetime was monitored at 1280 nm (10–13,18–20), using both a silicon cutoff filter and a monochromator.

RESULTS AND DISCUSSION

Although DCFH is extensively used as a probe of redox state, it has some chemical and photochemical characteristics that make it difficult to define its reactivity. For example, it is a good reducing agent, it can engage in photoinduced auto-oxidation reactions and the oxidation product is photoactive and absorbs visible light (2,9,21). Therefore, the mechanisms

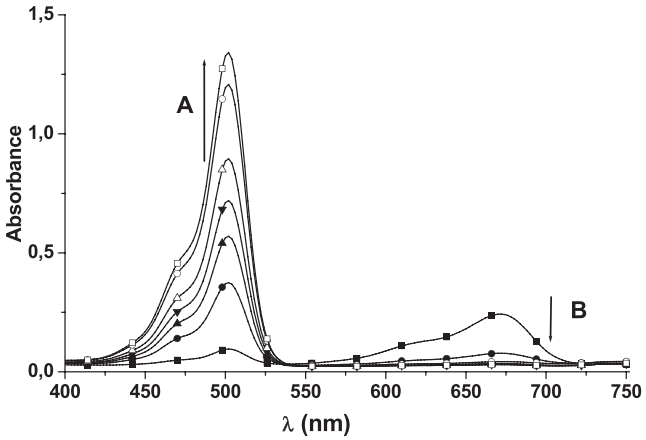

Figure 1. Absorption spectra of solutions containing 2′,7′-dichlorodihydrofluorescein (40 μM) and methylene blue (MB; 3 μM) after irradiation with a diode laser (50 mW) at λ = 664 nm. Spectra obtained from 0 (■) to 430 (□) s of irradiation in air-saturated solution. Formation of 2′,7′-dichlorofluorescein and photo-bleaching of MB are evident by following the absorption peaks at 502 nm (arrow A) and 664 nm (arrow B), respectively.

that lead to its oxidation may change depending on the type of excitation light and on the relative concentrations of species in the excited and ground states.

We will start by showing a condition in which DCFH seems not to react with $^1O_2$. Fig. 1 shows the absorption spectra of an aqueous solution containing 3 μM of MB (only MB monomers are present at such concentration) in the presence of 40 μM DCFH for several irradiation times (664 nm diode laser). One can observe the DCF formation, characterized by the increase in the absorption band at ~500 nm (arrow A). It is important to note that there is also a decrease in the MB absorption band at 664 nm (arrow B), indicating MB photobleaching, previously shown to result in the formation of the leuco-form (10,12,13). We also observed a six-fold increase in DCF formation rate by decreasing the concentration of molecular oxygen (20 min of nitrogen purging, data not shown) compared to the experiment performed in air-saturated solution. The presence of D_2O and azide shows little effects as reported earlier by Bilski *et al.* (9). However, instead of considering that DCFH does not react with $^1O_2$, we think that there is another explanation to such results. In fact, they can be simply understood if we consider that the irradiation of MB at 664 nm induces formation of MB triplets (10–13), which then react directly with DCFH molecules at this relatively large DCFH concentration, promoting its oxidation to DCF and the reduction of MB to MBH (a two electron plus proton reduction). The isosbestic point at 538 nm further substantiates this claim. Of note, the reduction of MB to MBH can only be explained by the consecutive oxidation of DCFH. The six-fold increase in the oxidation rate in the absence of oxygen agrees with this mechanism because oxygen deactivates triplet species, decreasing the efficiency of the reaction between reducing substrates (DCFH in this case) and triplets (22,23). The fact that azide and D_2O have no effect is also easily explained. Under these conditions DCFH molecules react directly with MB triplets, without the intermediacy of $^1O_2$. Other authors have also reported the oxidation of DCFH by direct reaction with sensitizers (9,21).

With the aim of investigating whether or not $^1O_2$ reacts with DCFH, we changed the experimental conditions decreasing the DCFH concentration (DCFH = 100 nM) to avoid its direct reaction with photosensitizer triplets, *i.e.* under this condition the rate of reaction between sensitizer triplets and oxygen is much larger than the rate of reaction with DCFH. Photochemistry of MB was utilized to generate specifically $^1O_2$ or $MB^{\bullet 2+}$ and $MB^{\bullet}/O_2^{\bullet -}$ and to test the reactivity of these species against DCFH.

Figure 2A shows the $^1O_2$ phosphorescence emission spectra in the presence of MB monomers (excess of micelles) and its absence in the presence of dimers, which leads to the formation of $MB^{\bullet 2+}$ and $MB^{\bullet}$ (10–13). The emission band centered at ~1280 nm with lifetime of 4.6 μs (Fig. 2A inset) is characteristic of $^1O_2$ in aqueous solutions (18–20). In the presence of dimers in air-saturated solutions, $MB^{\bullet}$ is transformed in $O_2^{\bullet -}$ in the microsecond time scale (10–13). $MB^{\bullet 2+}$ is a relatively stable cation radical (lifetime of 300 μs in water), which reacts with reducing agents, *i.e.* second-order rate constant with DABCO was measured to be 6×10^6 $M^{-1}s^{-1}$ (10). Figure 2B presents the corresponding DCF fluorescence emission spectra from the solutions described above. Note that the fluorescence intensity of the solution containing monomeric MB, which generates mainly $^1O_2$ under irradiation, increases considerably compared with nonirradiated samples, suggesting that DCFH does react with $^1O_2$. It is also interesting to note that in the conditions in which radical formation is favored (MB dimers), DCF oxidation is larger than in the solution in which only $^1O_2$ is generated.

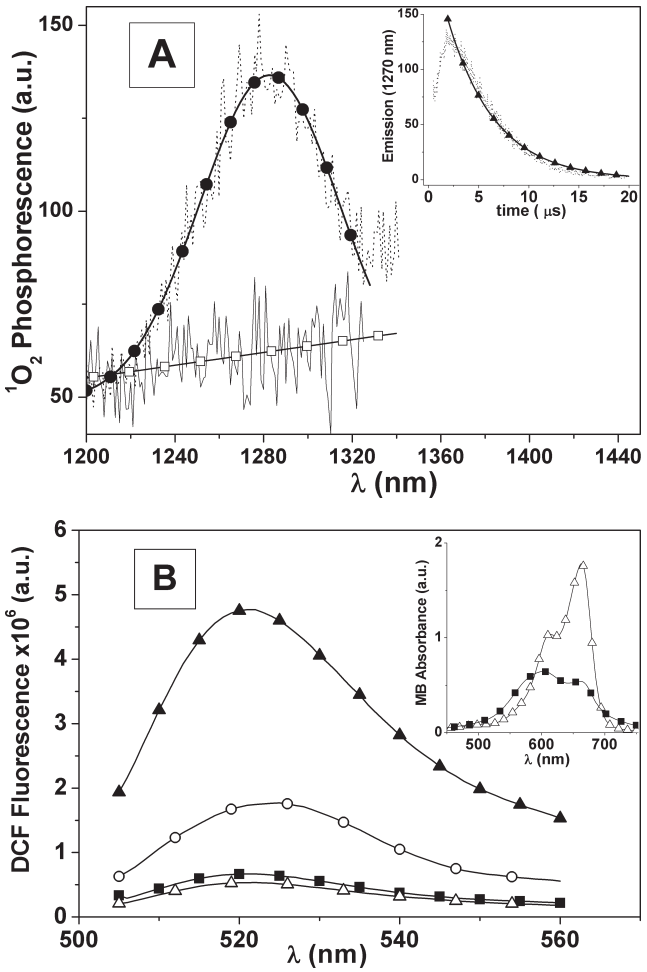

Figure 2. (A) $^1O_2$ phosphorescence emission of aqueous solution of MB = 20 μM in the presence of MB monomers (SDS = 30 mM, ●) and dimers (SDS = 1 mM, □) with DCFH = 100 nM. Inset: $^1O_2$ phosphorescence decay in the presence of MB monomers (MB = 20 μM, SDS = 30 mM, DCFH = 100 nM). The lifetime of 4.6 μs was calculated by an exponential fit (solid line). (B) DCF fluorescence emission (maximum at 522 nm) in aqueous solution (pH = 7) with DCFH = 100 nM and MB = 20 μM before irradiation in the monomeric (△, SDS = 30 mM) and dimeric (■, SDS = 1 mM) forms and after 3 min irradiation with 50 mW diode laser at λ = 664 nm, in the presence of MB dimers (▲) and monomers (○). Inset: absorption spectra of MB dimer (■) and monomer (△). The absorbance of all samples did not change during irradiation (λ_{exc} for DCF = 498 nm).

It is not expected that DCFH partitions in the micelle pseudo-phase because both DCFH and SDS have negative charges. MB partitions in the micelle pseudo-phase and because of the low MB concentrations (micromolar) compared to the SDS concentrations (millimolar), MB is totally bound in the micelles (10,12). Because the diffusion length of $^1O_2$ is much larger than the average size of the micelles, DCFH oxidation can occur in the bulk of the solution, where DCFH is preferentially located (24). The extent of the reaction of $^1O_2$ with a given target will depend on its local steady-state concentration and on the bimolecular rate constant (24).

In order to study the reaction of $^1O_2$ with DCFH, usual controls with deuterated water (D_2O) and sodium azide were performed. By following the increase in DCF fluorescence as a function of irradiation time in water and in D_2O, it is clear that the process of photo-oxidation is faster (rate of DCF formation in D_2O is ~3.6 times faster than in water) and more efficient (amount of formed DCF is three times larger) in D_2O (Fig. 3A). These results can be explained by the fact that the $^1O_2$ lifetime in water is *ca* 4.5 μs and in ~100% D_2O is about 35–40 μs (20). Assuming that no other interference occurs, the steady-state concentration of $^1O_2$ would be ~10 times larger giving consequently ~10 times faster reaction rate in ~100% D_2O. In our experiments we observed only ~3.6 times faster rate. However, it is important to emphasize that we were not using ~100% D_2O in our experiments because DCFH is prepared *in situ* by using aqueous NaOH solution. Furthermore, when the aqueous solution was irradiated in the presence of sodium azide (a known $^1O_2$ quencher), the DCF formation rate was ~5-fold reduced and the final DCF formation was three-fold decreased (Fig. 3B). Therefore, photo-oxidation of DCFH to DCF *via* $^1O_2$ indeed takes place and it was diminished by azide and increased in D_2O.

Another result that is in agreement with the reaction of $^1O_2$ with DCFH was obtained when the concentration of SDS was changed. It can be observed that the DCF formation rate increases with the increase in SDS concentration (Fig. 3C). Because the bulk solvent is the same (water) and the reactants are the same ($^1O_2$ and DCFH), the bimolecular rate should also be the same. Consequently, the steady-state concentration of $^1O_2$ must have increased with the increase in SDS concentration. Under this condition the rate of $^1O_2$ production depends on the oxygen concentration in the micelle microenvironment, which is increased with the increase in SDS concentration (10,12,24). In fact, we and others have observed increases in $^1O_2$ production with the increase in micelle

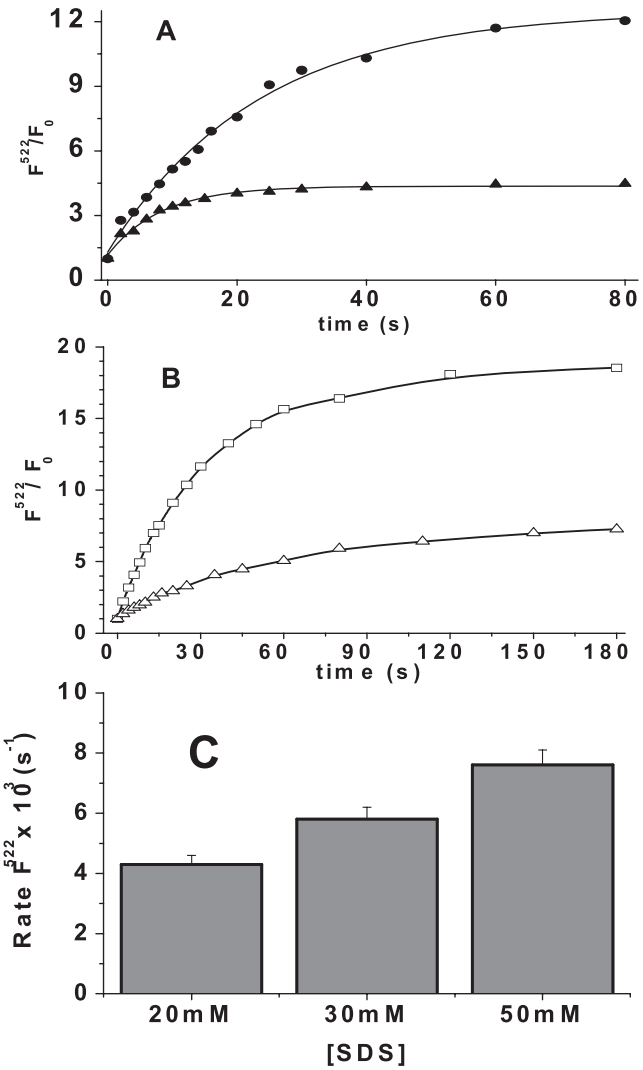

Figure 3. (A) Fluorescence increase (F^{522}/F_o) at 522 nm as a function of irradiation time in H_2O (▲) and D_2O (●). (B) Fluorescence increase at 522 nm as a function of irradiation time in aqueous solution in the presence (△) and absence (□) of 1.43 mM of sodium azide. (C) Rate of fluorescence increase in H_2O in the presence of 20, 30 and 50 mM of SDS. Irradiations with diode laser at λ = 664 nm, 50 mW and λ_{exc} of DCF = 498 nm, DCFH = 100 nM, MB = 3 μM.

concentration as well as with the increase in the molar fraction of the organic phase in other micro-heterogeneous systems (10,12,24), which can explain the results shown in Fig. 3C.

In the presence of monomers of MB, $O_2^{\bullet-}$ could also be formed by a side reaction leading to the reduction of $^1O_2$ (25). Although we had no reason to suspect that the reduction of $^1O_2$ was taking place in our experimental conditions, we tested the effect of SOD. It can be observed that in aqueous and in D_2O solutions both in the presence of SOD (Fig. 4A), the oxidation of DCFH follows profiles similar to that observed in the absence of SOD (Fig. 3A), *i.e.* rate of DCF formation is faster in D_2O, in agreement again with the oxidation by $^1O_2$. Therefore, these results confirm that $^1O_2$ reacts with DCFH. We believe that Bilski *et al.* (9) did not observe the reaction of DCFH with $^1O_2$ because of the large DCFH concentrations that were used, favoring the direct reaction with the photosensitizer triplets and because the rose bengal (RB)/DCFH system has a more complicated photochemistry due to the closer absorption bands of RB and DCF, which favor DCF auto-oxidation.

Although the main idea of this work was to test the reactivity of DCFH against $^1O_2$, the reactivity of DCFH against other species, including $O_2^{\bullet-}$, is also controversial (26–29). Some cell studies have indicated increased DCF formation in the presence of SOD (26). In cell-free extracts, presence (27) and absence (28) of reactivity against $O_2^{\bullet-}$ have been reported. In our system $O_2^{\bullet-}$ is generated directly in the presence of ground state dimers of MB. Experiments aiming to test the possible role of $O_2^{\bullet-}$ in the oxidation of DCFH were realized by testing the effect of SOD and catalase (Fig. 4B). It is clear that neither SOD nor SOD plus catalase shows any effect in the oxidation of DCFH. Therefore, our results also indicate that DCFH does not react with $O_2^{\bullet-}$, which is in agreement with other authors (2,26,28). The direct reaction with H_2O_2 has already been ruled out (2,21). This result also leads to the conclusion that the oxidizing species that is reacting with DCFH when ground state dimers are excited is the radical dication of MB ($MB^{\bullet 2+}$). Note that in this condition, besides $O_2^{\bullet-}$, $MB^{\bullet 2+}$ is the only species present in solution few microseconds after light excitation. Triplet species are deactivated in the nanosecond time scale (10,12). Several literature reports provide support to this interpretation—$MB^{\bullet 2+}$ reacts

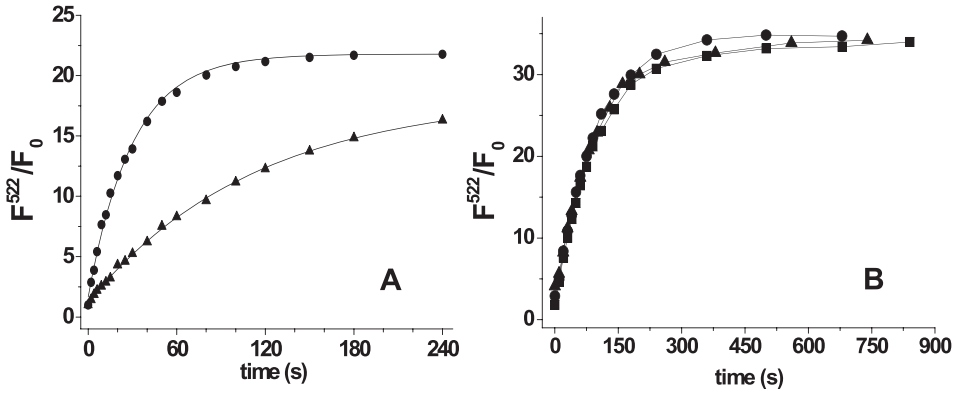

Figure 4. (A) Fluorescence increase (F^{522}/F_o) at 522 nm as a function of irradiation time in H_2O (▲) and D_2O (●) in the presence of SOD ~300 U mL^{-1}. (B) Fluorescence increase (F^{522}/F_o) at 522 nm as a function of irradiation time in H_2O in the presence of 1 mM SDS (~80% of MB dimers; Refs. [10,12]) (■), 300 U mL^{-1} of SOD (●) and 300 U mL^{-1} of SOD and 300 U mL^{-1} of catalase (▲), DCFH = 100 nM, MB = 5 μM.

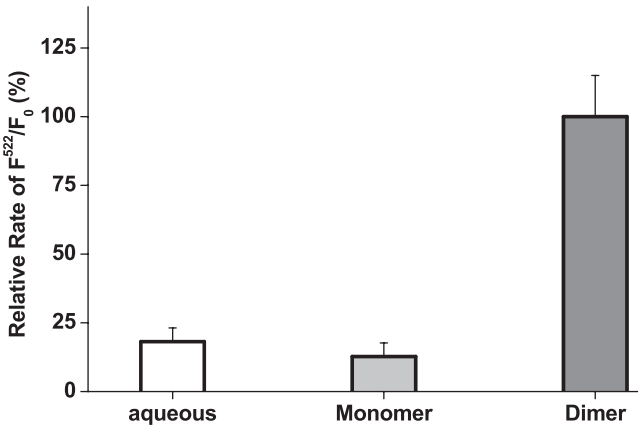

Figure 5. DCF fluorescence emission rate at 522 nm in the presence of MB in water solutions (dimers and monomers are present, [dimer] $< 1\%$), in 50 mM SDS where only monomers are present, and in 1 mM SDS where dimers are favored (dimer ~85%; Refs. [10,12]). Average and standard deviations of three independent measurements are shown. λ_{exc} = 498 nm, DCFH = 100 nM, MB = 10 μM.

with reducing agents like DABCO (10), the DCFH oxidizing species in cell studies are likely to be oxidizing radicals generated by peroxidases and DCFH reacts with compound I of horseradish peroxidase (30–32).

It is interesting to compare the reactivity of DCFH against either $^1O_2$ or $MB^{\bullet 2+}$. As mentioned before, in the condition in which $MB^{\bullet 2+}$ is formed (MB dimers are excited), DCF formation is faster and larger. Figure 5 shows the quantitative estimation of this effect. The rate of increase in DCF fluorescence is around 10 times larger in the presence of excess of dimers (1 mM SDS) than in the presence of monomers (50 mM SDS) and it is five times larger compared to that of water. As mentioned before, this rate should depend on the steady-state local concentration of reactant species and on bimolecular rate constants. Knowing that the efficiencies of either $^1O_2$ or $MB^{\bullet 2+}$ generations are similar in the presence of MB monomers or dimers (10), respectively, one could think, at first glance, that the reactivity of $MB^{\bullet 2+}$ against DCFH is larger than that of $^1O_2$. However, before reaching such a conclusion, it is important to consider the lifetimes of $MB^{\bullet 2+}$ and $^1O_2$ in solution. The rate constant for the spontaneous decay of $MB^{\bullet 2+}$ in aqueous solution is $\sim 3 \times 10^3$ s^{-1} (10), whereas for $^1O_2$ in water it is around $\sim 3 \times 10^5$ s^{-1} (20), *i.e.* $MB^{\bullet 2+}$ lives hundreds of microseconds, while $^1O_2$ lives only several microseconds. As a consequence, the steady-state concentration of $^1O_2$ is smaller than that of $MB^{\bullet 2+}$. Considering that the final concentration of oxidized DCF is only 1 order of magnitude larger in the condition in which $MB^{\bullet 2+}$ is generated compared to the condition in which $^1O_2$ is generated, one can conclude that $^1O_2$ is supposedly more reactive than $MB^{\bullet 2+}$ against DCFH. However, in order to obtain a quantitative estimate of the relative reactivity of $^1O_2$ and $MB^{\bullet 2+}$ against DCFH, it is necessary to realize a more detailed kinetic study in order to calculate the second-order rate constants. In cells in the case of redox misbalance caused by photoinduced processes, DCFH may help detect the generation of $^1O_2$. However, during photochemical reactions it is always important to also consider the DCFH oxidation by oxidizing radicals and triplet species (9,21).

The information that $^1O_2$ reacts efficiently with DCFH is especially important in the area of PDT, in which $^1O_2$ is generated *in situ* and the amount of ROS (especially $^1O_2$) is related to the efficiency of cell death (33,34). Researchers in other fields should also be aware that $^1O_2$ probably contributes to the total DCF fluorescence increase observed in cells even if $^1O_2$ is not directly generated (35,36).

CONCLUSIONS

Our results clearly show that DCFH can be directly oxidized by $^1O_2$ and $MB^{\bullet 2+}$. We suggest that the efficiency of the reaction of DCFH with $^1O_2$ is larger than that with $MB^{\bullet 2+}$. The fact that DCFH reacts with $^1O_2$, and with many other oxidizing agents, gives confidence that DCFH can continue to be used as a probe to monitor general redox misbalance in cells.

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REFERENCES

1. Sies, H. (1997) Oxidative stress: Oxidants and antioxidants. *Exp. Physiol.* **82**, 291–295.
2. Gomes, A., E. Fernandes and J. L. F. C. Lima (2005) Fluorescence probes used for detection of reactive oxygen species. *J. Biochem. Biophys. Methods* **65**, 45–80.
3. Soh, N. (2006) Recent advances in fluorescent probes for the detection of reactive oxygen species. *Anal. Bioanal. Chem.* **386**, 532–543.
4. Gomes, A., E. Fernandes and J. L. F. C. Lima (2006) Use of fluorescence probes for detection of reactive nitrogen species: A review. *J. Fluoresc.* **16**, 119–139.
5. Bartosz, G. (2006) Use of spectroscopic probes for detection of reactive oxygen species. *Clin. Chim. Acta* **368**, 53–76.
6. Royall, J. A. and H. Ischiropoulos (1993) Evaluation of 2′,7′-dichlorofluorescin and dihydrhorodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302**, 348–355.
7. Tobi, S. E., N. Paul and T. J. McMillan (2000) Glutathione modulates the level of free radicals produced in UVA-irradiated cells. *J. Photochem. Photobiol. B, Biol.* **57**, 102–112.
8. Bryska, M., A. Bacia and D. Elbaum (2001) Oxidative and hydrolytic properties of β-amyloid. *Eur. J. Biochem.* **268**, 3443–3454.
9. Bilski, P., A. G. Belanger and C. F. Chignell (2002) Photosensitized oxidation of 2′,7′-dichlorofluorescin: Singlet oxygen does not contribute to the formation of fluorescent oxidation product 2′,7′-dichlorofluorescein. *Free Radic. Biol. Med.* **33**, 938–946.
10. Junqueira, H. C., D. Severino, L. G. Dias, M. Gugliotti and M. S. Baptista (2002) Modulation of the methylene blue photochemical properties based on the adsorption at aqueous micelle interfaces. *Phys. Chem. Chem. Phys.* **4**, 2320–2328.
11. Estevam, M. L., O. R. Nascimento, M. S. Baptista, P. Di Mascio, A. Faljoni-Alario, M. R. Zucchi and I. L. Nantes (2004) Changes in the spin state and reactivity of cytochrome *c* induced by photochemically generated singlet oxygen and free radicals. *J. Biol. Chem.* **279**, 39214–39222.
12. Severino, D., H. C. Junqueira, M. Gugliotti, D. S. Gabrielli and M. S. Baptista (2003) Influence of negatively charged interfaces on the ground and excited state properties of methylene blue. *Photochem. Photobiol.* **77**, 459–468.
13. Gabrielli, D., E. Belisle, D. Severino, A. J. Kowaltowski and M. S. Baptista (2004) Binding, aggregation and photochemical properties of methylene blue in mitochondrial suspensions. *Photochem. Photobiol.* **79**, 227–232.

14. Tardivo, J. P., A. Del Giglio, C. S. de Oliveira, D. S. Gabrielli, H. C. Junqueira, D. B. Tada, D. Severino, R. F. Turchiello and M. S. Baptista (2006) Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications. *Photodiagn. Photodyn. Ther.* **2**, 165–238.
15. Tardivo, J. P., A. Del Giglio, L. H. Paschoal and M. S. Baptista (2006) A new PDT protocol to treat AIDS-related Kaposi's sarcoma. *Photomed. Laser Surg.* **24**, 528–531.
16. Orth, K., G. Beck, F. Genze and A. Ruck (2000) Methylene blue mediated photodynamic therapy in experimental colorectal tumors in mice. *J. Photochem. Photobiol. B, Biol.* **57**, 186–192.
17. Kolarova, H., R. Bajgar, K. Tomankova, P. Nevrelova and J. Mosinger (2007) Comparison of sensitizers by detecting reactive oxygen species after photodynamic reaction in vitro. *Toxicol. In Vitro* **21**, 1287–1291.
18. Cadenas, E. and H. Sies (1984) Low-level chemiluminescence as an indicator of singlet molecular oxygen in biological systems. *Methods Enzymol.* **105**, 221–231.
19. Bonnett, R. (2000) *Chemical Aspects of Photodynamic Therapy. Advanced Chemistry Texts*. Gordon & Breach Sci Publisher, UK.
20. Wilkinson, F., W. P. Helman and A. B. Ross (1995) Rate constants for the decay and reactions of the lowest electronically excited singlet-state of molecular-oxygen in solution—An expanded and revised compilation. *J. Phys. Chem. Ref. Data* **24**, 663–1021.
21. O'Mailley, Y. Q., K. Reszka and B. E. Britigan (2004) Direct oxidation of 2′,7′-dichlorodihydrofluorescein by pyocyanin and other redox-active compounds independent of reactive oxygen species production. *Free Radic. Biol. Med.* **36**, 90–100.
22. Baptista, M. S. and G. L. Indig (1998) Effect of BSA binding on photophysical and photochemical properties of triarylmethane dyes. *J. Phys. Chem. B* **102**, 4678–4688.
23. Baptista, M. S. and G. L. Indig (1997) Mechanism of photobleaching of ethyl violet non-covalently bound to bovine serum albumin. *Chem. Commun.* **18**, 1791–1792.
24. Lissi, E. A., M. V. Encinas, E. Lemp and M. A. Rublo (1993) Singlet oxygen $O_2(^1\triangle_g)$ bimolecular processes. Solvent and compartmentalization effects. *Chem. Rev.* **93**, 699–723.
25. Schweitzer, C. and R. Schmidt (2003) Physical mechanisms of generation and deactivation of singlet oxygen. *Chem. Rev.* **103**, 1685–1757.
26. Carter, W. O., P. K. Narayanan and J. P. Robinson (1994) Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J. Leukoc. Biol.* **55**, 253–258.
27. Zhu, H., G. L. Bannenberg, P. Moldéus and H. G. Shertzer (1994) Oxidation pathways for the intracellular probe 2′,7′-dichlorofluorescein. *Arch. Toxicol.* **68**, 582–587.
28. Hempel, S. L., G. R. Buettner, Y. Q. O'Malley, D. A. Wessels and D. M. Flaherty (1999) Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: Comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(6)-carboxy-2′,7′-dichlorodihydro fluorescein diacetate, and dihydrorhodamine 123. *Free Radic. Biol. Med.* **27**, 146–159.
29. LeBel, C. P., H. Ischiropoulos and S. C. Bondy (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **5**, 227–231.
30. Myhre, O., J. M. Andersen, H. Aarnes and F. Fonnum (2003) Evaluation of the probes 2′,7′-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem. Pharmacol.* **65**, 1575–1582.
31. Rota, C., C. F. Chignell and R. P. Mason (1999) Evidence for free radical formation during the oxidation of 2′,7′-dichlorofluorescin to the fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase: Possible implications for oxidative stress measurements. *Free Radic. Biol. Med.* **27**, 873–881.
32. Bonini, M. G., C. Rota, A. Tomasi and R. P. Mason (2006) The oxidation of 2′,7′-dichlorofluorescin to reactive oxygen species: A self-fulfilling prophesy? *Free Radic. Biol. Med.* **40**, 968–975.
33. Niedre, M. J., A. J. Secord, M. S. Patterson and B. C. Wilson (2003) *In vitro* tests of the validity of singlet oxygen luminescence measurements as a dose metric in photodynamic therapy. *Cancer Res.* **63**, 7986–7994.
34. Redmond, R. W. and I. E. Kochevar (2006) Spatially resolved cellular responses to singlet oxygen. *Photochem. Photobiol.* **82**, 1178–1186.
35. Holevinsky, K. O. and D. J. Nelson (1995) Simultaneous detection of free radical release and membrane current during phagocytosis. *J. Biol. Chem.* **270**, 8328–8336.
36. Gueven, N., O. J. Becherel, O. Howe, P. Chen, J.-F. Haince, M.-E. Ouellet, G. G. Poirier, N. Waterhouse, M. Fusser, B. Epe, J. M. Murcia, G. Murcia, C. H. McGowan, R. Parton, C. Mothersill, P. Grattan-Smith and M. F. Lavin (2007) A novel form of ataxia oculomotor apraxia characterized by oxidative stress and apoptosis resistance. *Cell Death Differ.* **14**, 1149–1161.