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Original Contribution

DIHYDROFLUORESCEIN DIACETATE IS SUPERIOR FOR DETECTING INTRACELLULAR OXIDANTS: COMPARISON WITH 2′,7′-DICHLORODIHYDROFLUORESCEIN DIACETATE, 5(AND 6)-CARBOXY-2′,7′-DICHLORODIHYDROFLUORESCEIN DIACETATE, AND DIHYDRORHODAMINE 123

Stephen L. Hempel*[,†] Garry R. Buettner,[†] Yunxia Q. O'Malley,[‡] Duane A. Wessels,* and Dawn M. Flaherty[‡]

*Department of Internal Medicine, Department of Veterans Affairs Medical Center and Departments of [‡]Internal Medicine and [†]Radiology, University of Iowa, Iowa City, IA, USA

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Abstract—To detect intracellular oxidant formation during reoxygenation of anoxic endothelium, the oxidant-sensing fluorescent probes, 2′,7′-dichlorodihydrofluorescein diacetate, dihydrorhodamine 123, or 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate were added to human umbilical vein endothelial cells during reoxygenation. None of these fluorescent probes were able to differentiate the controls from the reoxygenated cells in the confocal microscope. However, dihydrofluorescein diacetate demonstrated fluorescence of linear structures, consistent with mitochondria, in reoxygenated endothelium. This work tests the hypothesis that dihydrofluorescein diacetate is a better fluorescent probe for detecting intracellular oxidants because it is more reactive toward specific oxidizing species. To investigate this, dihydrofluorescein diacetate was exposed to various oxidizing species (hydrogen peroxide, superoxide [KO_2], peroxynitrite, nitric oxide, horseradish peroxidase, ferric iron, xanthine oxidase, cytochrome c, and lipoxygenase) and compared with the three other popular probes. Though oxidized dihydrofluorescein has higher molar fluorescence, comparison of the reactions of dihydrofluorescein with these other three probes in a cell-free system indicates that dihydrofluorescein is sometimes less fluorescent than the other probes. In addition, we find that the reactivity of all of the probes is very complex. Based on the results reported here, it is no longer appropriate to think of these probes as detecting a specific oxidizing species in cells, such as H_2O_2, but rather as detectors of a broad range of oxidizing reactions that may be increased during intracellular oxidant stress. Cell-loading studies indicate that dihydrofluorescein achieves higher intracellular concentrations than the second brightest intracellular probe, 2′,7′-dichlorodihydrofluorescein. This fact and its higher molar fluorescence may account for the superior brightness of dihydrofluorescein diacetate. Dihydrofluorescein diacetate may be a superior fluorescent probe for many cell-based studies. © 1999 Elsevier Science Inc.

Keywords—Iron, Peroxide, Nitric oxide, Peroxynitrite, Reactive oxygen species, Free radicals

INTRODUCTION

The detection of H_2O_2 by 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA), (Fig. 1A), in a cell-free system [1,2] generated interest in the use of fluorescent probes to detect cell-derived oxidants. Shortly after publication of Keston and Brandt's papers [1,2], DCHF-DA was used as a detector of intracellular oxidants [3]. In these early studies, the acetate groups were hydrolyzed with base before adding to the cells [3]. Fluorescence was then detected in cell lysates [3]. Cell biologists did not use DCHF-DA to detect intracellular oxidants in live cells until 1983 when Bass demonstrated that DCHF-DA could be used with flow cytometry to detect oxidant formation by activated neutrophils [4]. Bass speculated

Address correspondence to: Stephen L. Hempel, Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA; Tel: (319) 356-8265; Fax: (319) 353-6406;
E-Mail: stephen-hempel@uiowa.edu.

Dr. Hempel has a primary appointment in the Department of Internal Medicine, Department of Veterans Affairs Medical Center and The University of Iowa, Iowa City, IA, and a secondary scientific appointment in the Department of Radiology, The University of Iowa, Iowa City, IA.

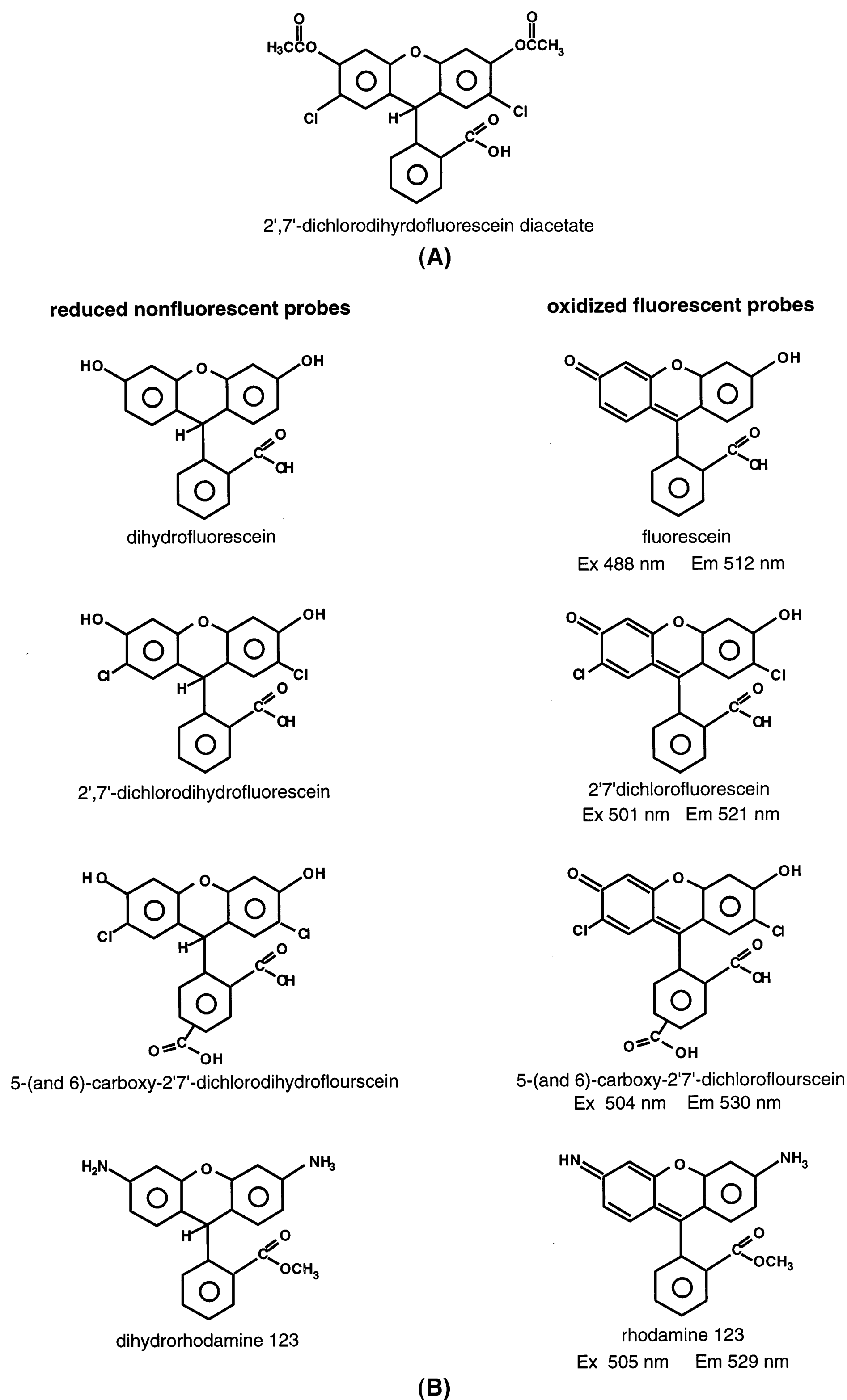


Fig. 1. Structures of common oxidant-sensitive fluorescent probes. (A) 2′,7′-dichlorodihydrofluorescein diacetate. (B) Reduced and oxidized structures of four common fluorescent probes. The nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate is stable in ethanol and unreactive toward oxidants. After base hydrolysis of the ester bonds, releasing the acetate groups, the resulting compound, 2′,7′-dichlorodihydrofluorescein, is reactive toward oxidizing species, such as peroxide, in the presence of appropriate cofactor(s), such as horseradish peroxidase [2]. The nomenclature is as follows: 2′,7′-dichlorofluorescin diacetate or 2′,7′-dichlorodihydrofluorescein

that the probe diffuses into the cell, intracellular esterases hydrolyze the acetate groups, and the resulting 2′,7′-dichlorofluorescin (DCHF) then reacts with intracellular oxidants resulting in the observed fluorescence [4]. Since then, there have been numerous reports using DCHF-DA to measure intracellular oxidants. Much of this work has used flow cytometry [4–6] and cells capable of a respiratory burst, such as macrophages or polymorphonuclear leukocytes [4–7]. More recently, investigators have used DCHF-DA to study other cells, such as the endothelial cell [8,9]. Additional probes have also been introduced. Dihydrorhodamine 123 (DHR123), (Fig. 1B), is presumed to localize to mitochondria [10], whereas 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (5&6DH-DA), (Fig. 1B), is supposed to be retained in the cell better than DCHF, thereby yielding more fluorescence [11,12].

Initially, three intracellular fluorescent probes (DCHF-DA, DHR123, and 5&6DH-DA) were used in our laboratory as a tool to detect intracellular oxidants formed during reoxygenation of anoxic endothelial cells [13–17]. We speculated that the mitochondria would be a likely source of oxidants during reoxygenation [17] and attempted to use confocal microscopy with these fluorescent probes to detect mitochondria-derived species during reoxygenation. However, none of these probes gave a fluorescent signal that was significantly different than the room air controls. Subsequently, we found that dihydrofluorescein diacetate (HFLUOR-DA) readily imaged linear intracellular structures having the appearance of mitochondria during reoxygenation of the endothelium. These findings indicate that HFLUOR-DA, first described as fluorescein in 1871, may be a useful tool for other investigators studying intracellular oxidants [18,19].

The present study tests the hypothesis that HFLUOR-DA is superior for detecting intracellular oxidants by confocal microscopy because it is more reactive toward oxidants than the other three probes. Our results indicate that the superiority of HFLUOR-DA for imaging intracellular oxidants by confocal microscopy is not due simply to enhanced reactivity. We find that the reactivity of all four probes is very complex and each probe has a unique reactivity toward specific oxidizing species. Based on results reported here, it is no longer appropriate to think of these probes as detecting a specific species, such as H_2O_2 [6,8], but rather as detectors of a broad range of oxidizing reactions that may be increased during intracellular oxidant stress [20]. One explanation for the increased fluorescence of HFLUOR-DA, compared with the other fluorescent probes, is a higher molar fluorescence. In addition, HFLUOR achieves higher intracellular concentrations than DCHF, thus more compound is available for oxidation to fluorescent species.

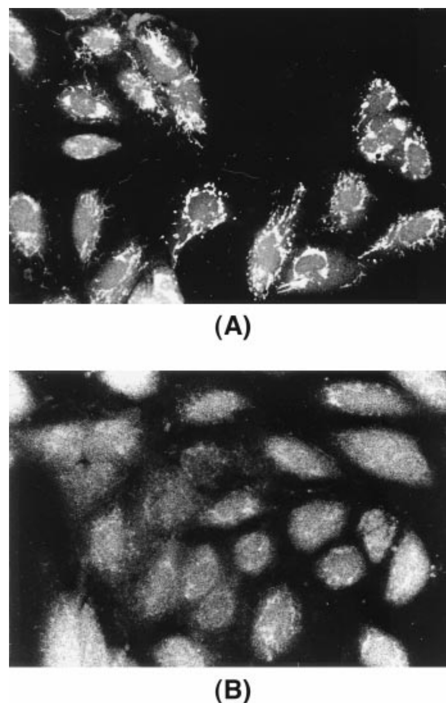


Fig. 2. Dihydrofluorescein identifies linear structures during reoxygenation of anoxic endothelium. Human umbilical vein endothelial cells were anoxic for 24 h in an atmosphere of 95% N_2 and 5% CO_2 at 37°C, then reoxygenated (A) for 30 min with HBSS containing 20 μM fluorescein diacetate at ambient oxygen tension, 25°C. (B) Control cells were cultured 24 h at room-air oxygen tension plus 5% CO_2 then loaded with 20 μM dihydrofluorescein diacetate in HBSS. Images were generated by confocal microscopy using a water immersion lens. Excitation was 488 nm and emission was detected at 521 nm. The linear structures visualized in the reoxygenated cells are characteristic of mitochondria.

MATERIALS AND METHODS

Reagents

2′,7′-dichlorodihydrofluorescein diacetate (#D399), 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (#C-400), dihydrorhodamine 123 (#D632), dihydrofluorescein diacetate (#D1194), 5(and 6)-carboxy-2′,7′-dichlorofluorescein (#C368), and rhodamine 123

Fig. 1. *Continued.* diacetate are equivalent names of the unreactive and nonfluorescent reduced compound. 2′,7′-dichlorodihydrofluorescein or 2′,7′-dichlorofluorescin are equivalent names of the nonfluorescent reduced compound. 2′,7′-dichlorofluorescein is the oxidized fluorescent compound. The maximum excitation and emission wavelengths are shown below the structures of the oxidized probes. The excitation and emission wavelength will vary slightly based on pH and solvent system.

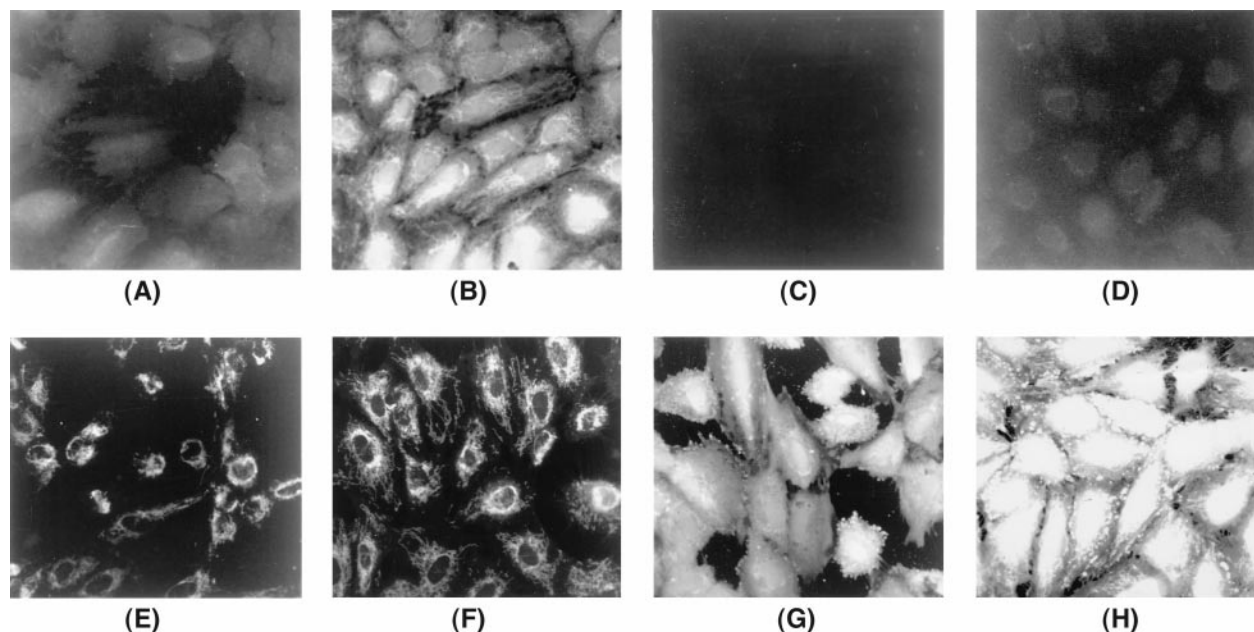


Fig. 3. Comparison of four probes by confocal microscopy. Human umbilical vein endothelial cells were loaded with 100 μM menadione for 60 min in the presence of 20 μM of different fluorescent probes in HBSS at 25°C. The cells were imaged at precisely 60 min using an iris setting of 3 and a gain of 1000. The control cells were loaded with the same probe under the same conditions but without menadione. The microscope settings are the same for each panel such that the pixels in Panel H are saturated and little cellular detail is apparent. At lower gain settings (not shown) more cellular detail is apparent in Panel H, as in Fig. 2. (A) DCHF, control. (B) DCHF, menadione. (C) 5&6DH, control. (D) 5&6DH, menadione. (E) DHR123, control. (F) DHR123, menadione. (G) HFLUOR, control. (H) HFLUOR, menadione.

(#R302) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Powdered medium 199 (with Earle's salts and L-glutamine but without $NaHCO_3$), endothelial growth factor (#E2759), reduced glutathione (#G4251), glutathione peroxidase (#G6137), horseradish peroxidase, type I (#P8125), xanthine oxidase (#X1875), soybean lipoxygenase (#L6632), catalase (#C9322), Cu/Zn-SOD (#S2515), fluorescein (#F7505) and 2′,7′-dichlorofluorescein (#D9053) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); fetal bovine serum (FBS) was purchased from Hy-Clone (Logan, UT, USA); H_2O_2 30% solution from Fisher Scientific (Fair Lawn, New Jersey), and human fibronectin from Collaborative Research (Bedford, MA, USA). All other chemicals were reagent grade.

Solutions

Medium 199 (M199) contained 9.87 g/l medium 199 powder plus 2.2 g/l $NaHCO_3$, 100,000 U/l penicillin G, 100 mg/l streptomycin, 300 mg/l L-glutamine, plus 10% FBS, pH 7.4. HBSS contained 4.2 mM $NaHCO_3$, pH 7.4. $FeSO_4$ (6 mM final concentration) in 0.01 N HCl was prepared fresh daily from 18 MΩ water and purged with argon for 15 min. This solution was stored by sealing it under argon until use. In experiments that had no added Fe^{2+}, an equal volume of 0.01 N HCl argon-purged H_2O was added to cells to serve as a control. H_2O_2 was mixed with phosphate-buffered saline (PBS) (9.5 mM phosphate), pH 7.4, before each experiment. In spite of cold storage in the dark, there was some decay of the stock 30% H_2O_2 solution, so the concentration was adjusted by absorption spectroscopy according to the formula: $\varepsilon_{230} = 88$ l mole^{-1}cm^{-1}. PBS was stored over Chelex-100 beads to decrease adventious iron [21]. All concentrations quoted in the results and figure captions are final concentrations.

Peroxynitrite solution

Five milliliters of 0.6 M sodium nitrite in water was added to 5 ml 0.6 M hydrogen peroxide in acidified water (0.8 N HCl), then immediately (almost simultaneously) were added 5 ml 1.4 M sodium hydroxide in a well-stirred solution. MnO_2 was then added to remove excess H_2O_2. The resulting solution was filtered twice through a 0.2 micron Millipore (Bedford, MA, USA) filter with a syringe. The solution was then stored overnight at −20°C. The peroxynitrite that rises to the top is removed and the concentration is determined from the equation $\varepsilon_{302} = 1670$ M^{-1}cm^{-1} [22].

Nitric oxide solution

A saturated solution of $NO^{\bullet}$ was generated by the reaction of HCl with $NaNO_2$. A 1 M solution of $NaNO_2$ was deoxygenated by bubbling with N_2 in a closed jar with a vented headspace. After purging, a syringe was connected to the headspace vent. Through a separate port, small aliquots of deoxygenated 1 N HCl were added to the $NaNO_2$ solution with shaking. HCl injections were repeated until no more $NO^{\bullet}$ was produced. The $NO^{\bullet}$ gas in the headspace was then transferred to another closed jar containing deionized water purged with N_2. The concentration of the $NO^{\bullet}$-saturated solution in pure water is temperature dependent (2.10 mM at 20°C; 1.93 mM at 25°C).

Potassium superoxide

Reactions with KO_2 were performed during conditions of very low relative humidity. KO_2 aliquots were weighed carefully on a Cahn 21 microanalytic balance immediately before use. Solutions containing all of the reagents were added with vigorous mixing in a test tube containing KO_2. Five minutes after reacting with KO_2 the fluorescence was determined.

Endothelial culture

Human umbilical vein endothelial cells were isolated from fresh human umbilical cords and cultured in M199 with 10% FBS [16,23]. Cells were seeded on human fibronectin-coated 100-mm dishes at a density of 4×10^6 cells per dish. The medium was changed after 2–4 h to remove red blood cells and nonadherent tissue cells. Previous studies have shown that this technique yields cultures of high purity with minimal variability in cell number or total protein from well to well [16,24]. Cells were passed at a 1:3 split to 35-mm dishes on day 4 and 50 μg/ml endothelial cell growth factor was added. Cultures were examined by phase contrast microscopy prior to use to verify confluence and culture purity.

Confocal microscopy

Human umbilical vein endothelial cells were grown to confluence in 35-mm fibronectin-coated dishes. One hour prior to confocal imaging cells were washed with HBSS and then 20 μM fluorescent probe in HBSS was added to the cells. A BioRad MRC-600 Confocal microscope with an argon-krypton laser was used to obtain images. Cells were imaged via the epifluorescence mode with a 40× immersion lens with the following parameters: fluorescence transmission for fluorescein isothiocyanate, laser power 100%, gain 865, iris 3.0, black level 0, emission filter 521. Images were stored on a Motorola StarMax computer using the public domain National Institutes of Health Image program.

Anoxia-reoxygenation

Human umbilical vein endothelial cells were cultured in a special chamber flushed with 95% N_2 and 5% CO_2 for 24 h [16,24]. After the period of anoxia, cells were reoxygenated by washing with HBSS at ambient pO_2. Then 20 μM fluorescent probe was added for 30 min before confocal imaging.

Fluorescence spectrophotometry

In the cell-free experiments a computer-controlled Perkin-Elmer LS-50B luminescence spectrometer measured changes in fluorescence of the four probes. The unit was programmed to obtain readings at regular time intervals, which varied depending on the experiment. Concentrations of the fluorescent probes were confirmed using absorbance spectroscopy according to the extinction coefficients provided by the supplier. DCHF-DA, HFLUOR-DA, and 5&6DH-DA were hydrolyzed with base, 0.01 N NaOH for 30 min in the dark, then diluted with PBS to a working stock of 100 μM. DHR123 was dissolved in PBS and diluted to a working stock of 100 μM. Reagents were added in order as depicted in the Figs. 5–15 captions. Each probe was used at a final concentration of 5 μM for the cell-free studies. Because the baseline fluorescence varied from probe to probe and from experiment to experiment, the data presented represent the change from baseline (i.e., the baseline fluorescence is subtracted from all values). The Exλ was 488 nm and the Emλ was 521 nm, the same as the excitation and emission wavelengths of the confocal microscope. All fluorescence measurements were performed on room temperature solutions at ambient gas tension.

Cell fluorescence

Human umbilical vein endothelium at first passage were cultured to confluence on 48 well plates. Cells were washed with PBS, then PBS with glucose, pH 7.4, was added to the cells. DCHF-DA, HFLUOR-DA, DHR123, and 5&6DH-DA were added at a final concentration of 20 μM. Wells without cells were also loaded with fluorescent probes to serve as controls. Fluorescence measurements were made on a Fluostar 403 (BMG Lab Technologies, Durham, NC, USA) fluorescent platereader every 10 min at Exλ 488 nm and Emλ 510 (bandpass 5 nm) and 538 nm (bandpass 10 nm). The

Emλ 510 nm and Emλ 538 nm values were added together.

Molar fluorescence

Reagent grade oxidized probes were dissolved in methanol to a working stock of 100 μM, then diluted in 50 mM potassium-phosphate buffer, pH 9.0, to a final concentration of 0.5 μM. Concentrations were adjusted according to the extinction coefficients supplied by the manufacturer: 2′,7′-dichlorofluorescein, 91,000 $cm^{-1}M^{-1}$ at 502 nm; fluorescein, 98,700 $cm^{-1}M^{-1}$ at 490 nm; rhodamine 123, 95,900 $cm^{-1}M^{-1}$ at 507 nm (in methanol); and 5&6DH, 80,300 $cm^{-1}M^{-1}$ at 504 nm.

RESULTS

Confocal microscopy

Using three popular fluorescent probes, attempts to image mitochondria by confocal microscopy during reoxygenation of anoxic endothelium were unsuccessful (data not shown). DCHF-DA exhibited high background fluorescence in the control cells and little apparent increase in the reoxygenated cells. DHR123 exhibited bright mitochondria fluorescence in the control cells as well as the reoxygenated cells and 5&6DH-DA gave a low signal in the controls and the anoxia-reoxygenation

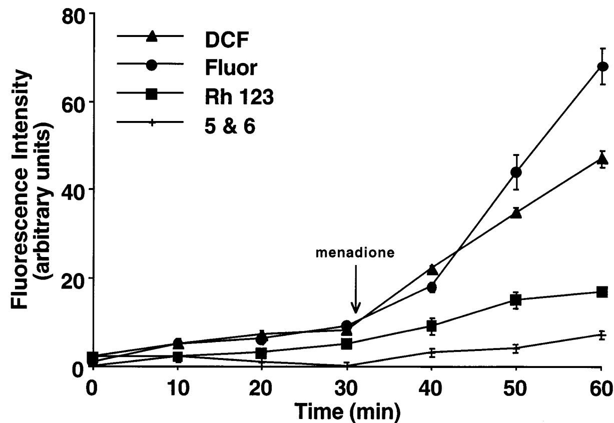


Fig. 4. Comparison of four probes by quantitative cell fluorescence. Human umbilical vein endothelial cells on 48 well plates were loaded with 20 μM (final) of the four fluorescent probes at 25°C starting at time zero as shown on the x axis. Fluorescence was determined by a fluorescence plate reader, y axis. Just after the 30 min fluorescence reading, 100 μM menadione was added (arrow). Cell-free wells with the same probes and menadione were used as negative controls. Only DHR123 in the cell-free wells had some initial fluorescence (52 arbitrary units) and slowly increased over 1 h (additional 19 arbitrary units, data not shown). These cell-free values were subtracted from the values in the figure. The oxidized probes depicted in the legend are 2′,7′-dichlorofluorescein (DCF), fluorescein (Fluor), rhodamine 123 (Rh 123), and 5(and 6)-carboxy-2′,7′-dichlorofluorescein (5 & 6); $n = 6$, ± SD.

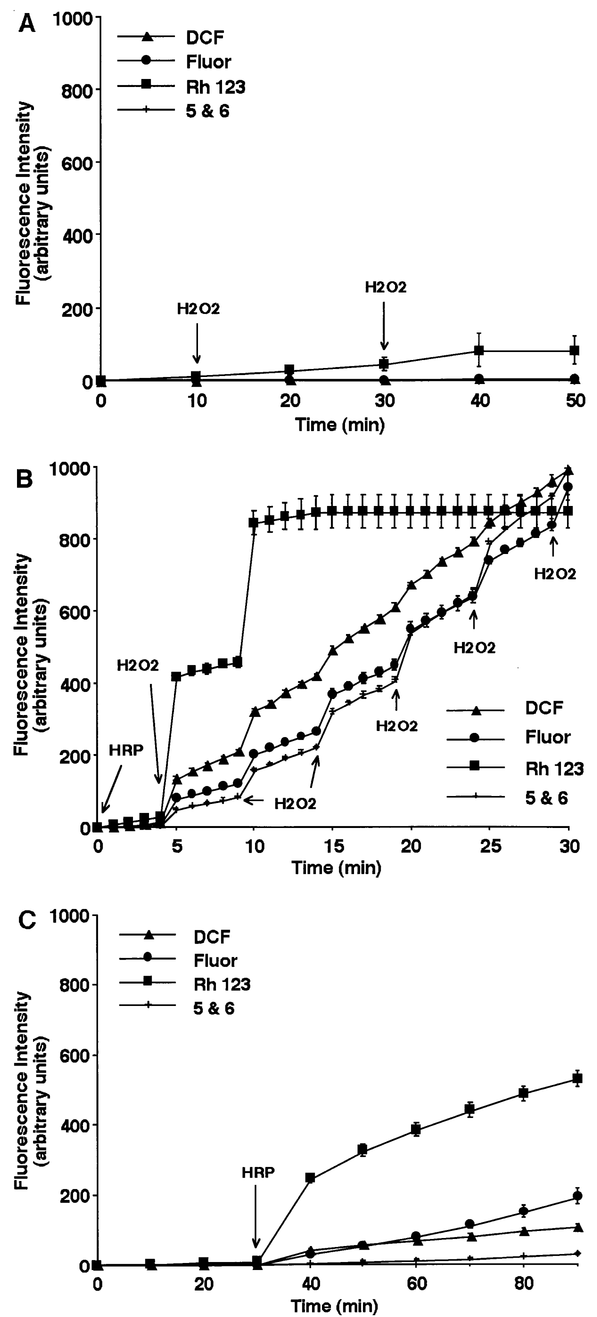


Fig. 5. Cofactors are necessary for the oxidation of fluorescent probes by peroxide. 5 μM of each probe were added to PBS, pH 7.5, and reagents added as described. Fluorescence was determined by a fluorescence spectrophotometer. The excitation wavelength was set to 488 nm and the fluorescent emission frequency was monitored at 521 nm, to conform to the wavelengths of the confocal microscope. The three probes requiring base hydrolysis, 2′,7′-dichlorodihydrofluorescein diacetate, dihydrofluorescein diacetate, and 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, were treated in the dark with 0.01 N NaOH for 30 min. In each panel, $n = 4$, ± SD. (A) At the arrows, 10 μM H_2O_2 was added to each of the probe solutions. (B) 30 U/ml horseradish peroxidase (HRP) were added, then 0.1 μM H_2O_2 at each arrow point. Because the background fluorescence at time 0 min is subtracted from each point, the absolute intensity of Rh 123 actually exceeds the full scale (1000) of the LS50B luminescence spectrometer. (C) 30 U/ml of HRP were added to each probe.

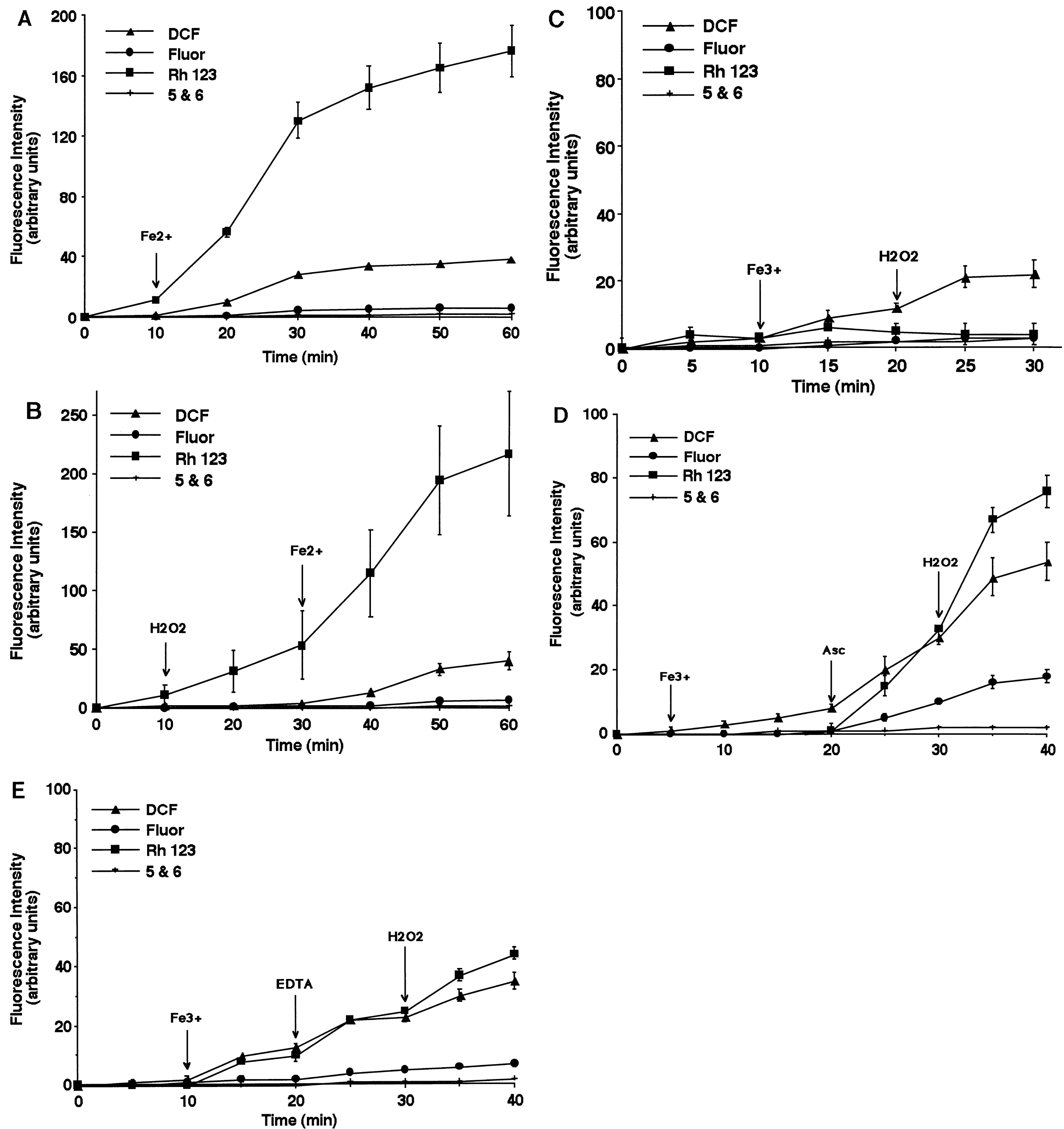


Fig. 6. Fe^{2+} oxidizes fluorescent probes without peroxide and acts as a peroxide cofactor. (A) 20 μM of Fe^{2+}, as $FeSO_4$, was added at 10 min. (B) 20 μM H_2O_2 was added first then 20 μM Fe^{2+}. (C) Fe^{3+} was added, then H_2O_2. (D) Fe^{3+}, then 100 μM ascorbate (Asc), then H_2O_2 were added. (E) Fe^{3+}, then 25 μM EDTA, then H_2O_2 were added (n = 4, ± SD).

cells. However, dihydrofluorescein diacetate (HFLUOR-DA) readily imaged linear structures consistent with mitochondria during reoxygenation of anoxic endothelium (Fig. 2). To determine if HFLUOR-DA gives superior results with another oxidant stress, the redox cycling agent menadione was tested. Cells were loaded with DCHF-DA, 5&6DH-DA, DHR123, or HFLUOR-DA at room temperature for 1 h in the presence of menadione, then imaged by confocal microscopy. Cell fluorescence is dependent on loading time of the fluorescent probes; therefore, these times were carefully controlled prior to obtaining the images. As shown in Fig. 3, HFLUOR-DA gives the brightest images in the control cells (panel G) and the cells treated with menadione (panel H). The

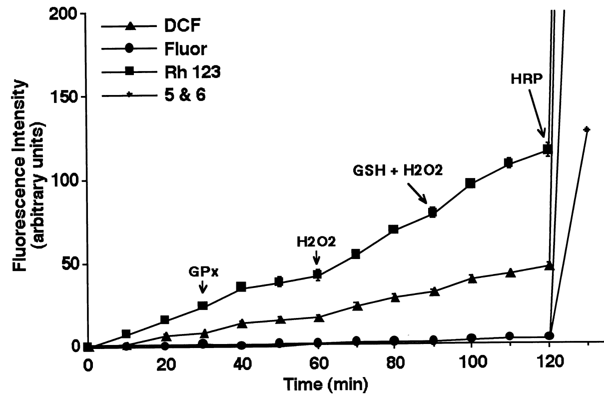
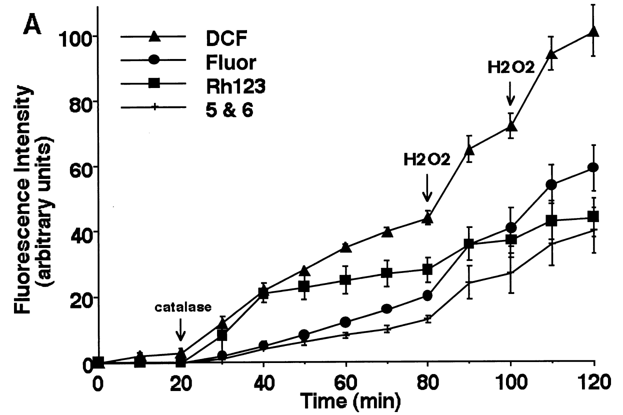
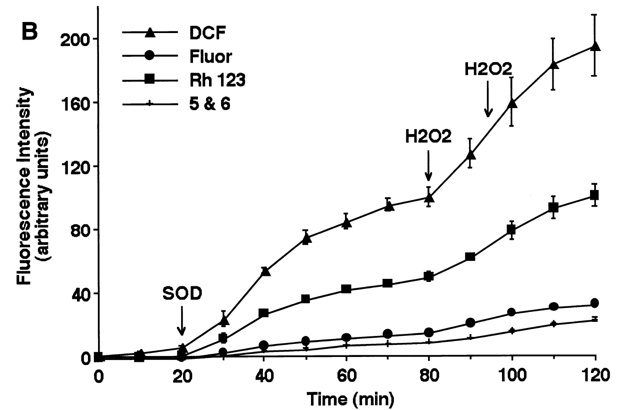


Fig. 7. Glutathione peroxidase is unreactive toward the fluorescent probes. Glutathione peroxidase, 30 U/ml, was added at 30 min. At 60 min, 1 μM H_2O_2 was added. At 90 min, 5 μM glutathione (GSH) was added concurrent with 1 μM H_2O_2. At 120 min, HRP, 30 U/ml, was added (n = 4, ± SD).

microscope settings are the same for each panels A–H such that the pixels in panel H are saturated and little cellular detail is apparent (compare with Fig. 2). DHR123 readily identifies linear structures consistent with mitochondria in the control cells and the cells treated with menadione, whereas 5&6DH-DA shows weak fluorescence. These results show important differences between these probes when used for confocal microscopy of endothelial cells.

These observations were confirmed using a fluorescence plate reader. The four probes were added to endothelial cells on 48 well plates. Additional wells without cells were used to measure background fluorescence. Fluorescent measurements were made every 10 min. After the 30 min measurement menadione was added (Fig. 4). These results demonstrate essentially no fluorescence from the cell-free wells containing DCHF-DA, HFLUOR-DA, or 5&6DH-DA (data not shown). DHR123 in the cell-free wells had some initial fluorescence and slowly increased over 1 h. The cell-free background readings were subtracted from the cell-based fluorescence shown in Fig. 4. These results reinforce the observations of Fig. 3, demonstrating the increased fluorescence of HFLUOR-DA in cell-based experiments. In addition, the findings in the cell-free control wells confirm that the diacetate-containing probes require interaction with cells to become fluorescent.





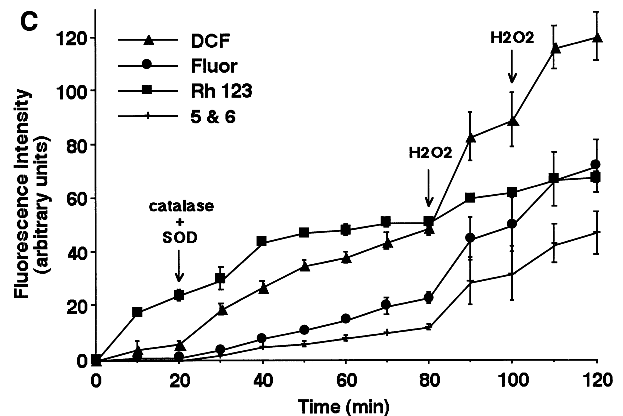


Fig. 8. Catalase and Cu/Zn-SOD oxidize fluorescent probes and act as cofactors in the reaction with H_2O_2. (A) At the arrow, 50 U/ml catalase were added. H_2O_2 10 μM is added at the arrows. (B) 5 U/ml Cu/Zn-SOD were added at the arrow, then 10 μM H_2O_2 at the arrows. (C) 5 U/ml Cu/Zn-SOD plus 50 U/mL catalase were added at the arrow, then 10 μM H_2O_2 at the arrows (n = 4, ± SD).

Requirement for cofactors

To test the hypothesis that dihydrofluorescein (HFLUOR) is brighter because it is more reactive toward oxidants, a series of cell-free experiments were performed comparing HFLUOR to the three other probes. The acetate groups were hydrolyzed with base, then the probes were oxidized by: H_2O_2, H_2O_2 plus horseradish peroxidase (HRP), or HRP alone (Fig. 5). The spontaneous rate of aerobic oxidation of all of the probes is low (Fig. 5A), 0–10 min. DHR123 does oxidize spontaneously in aerated buffer, but only to 3% of full scale in 30 min (data not shown). DHR123 was the most easily

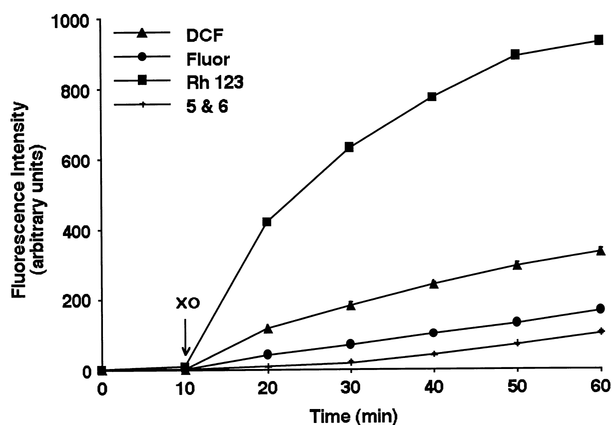


Fig. 9. Xanthine oxidase oxidizes the fluorescent probes. After 10 min observation, 20 mU/ml xanthine oxidase were added to all solutions as denoted by the arrow ($n = 4$, $\pm$ SD).

oxidized by H_2O_2 (Fig. 5A), but oxidizes less than 10% of full scale at 50 min. In the presence of HRP all of the probes are oxidized by H_2O_2; DHR123 is the most reactive, exceeding the full scale of the fluorometer (Fig. 5B). Note that after several additions of H_2O_2 in the presence of HRP, DCHF became unresponsive to additional aliquots of H_2O_2 (Fig. 5B). HRP alone oxidized all of the probes, HFLUOR being only slightly more reactive than the others (Fig. 5C). These results demonstrate the requirement of the fluorescent probes for a specific cofactor, such as HRP [2], for optimum detection of H_2O_2. The results also demonstrate that HRP alone may oxidize the probes to fluorescence in the absence of added H_2O_2 [9,20].

A previous report suggests that Fe^{2+} does not oxidize DCHF to fluorescence [20]. However, Fe^{2+} in the presence of oxygen forms reactive oxidants [25]. Therefore, the oxidation of the probes in the presence of Fe^{2+} using a longer period of reaction than in the previous study was tested [20]. DCHF, HFLUOR, and DHR123 are oxidized to fluorescence by Fe^{2+}, 5&6DH is not (Fig. 6A). H_2O_2 by itself causes little increase in fluorescence (Fig. 6B). However, the addition of Fe^{2+} to H_2O_2 causes a rapid increase in fluorescence. Again, 5&6DH has a small increase. The same experiment in Fig. 6B was repeated using *tert*-butylhydroperoxide. This resulted in the same rank order of reactivity, but all of the probes were less reactive with *tert*-butylhydroperoxide (data not shown). Fe^{3+} by itself, causes little increase in fluorescence and fluorescence does not increase after adding peroxide (Fig. 6C). Fe^{3+} plus ascorbate, which reduces Fe^{3+} to Fe^{2+}, increases the oxidation of the probes, again with the exception of 5&6DH (Fig. 6D). Fe^{3+} plus EDTA plus H_2O_2 causes a modest increase in fluorescence of DHR123 and DCHF, although there is little reaction with 5&6DH or HFLUOR (Fig. 6E). These results indicate that Fe^{2+} plus H_2O_2 and Fe^{2+} by itself oxidizes three probes to fluorescence; 5&6DH is the exception. Cofactors such as ascorbate or EDTA may enhance the reactivity of Fe^{3+} with DCHF and DHR123 as previously reported [26]. However, HFLUOR is less reactive under these conditions and 5&6DH is essentially not reactive.

Other intracellular cofactors

Consistent with our results, previous authors have demonstrated that H_2O_2 does not readily react with the fluorescent probes without an appropriate cofactor, such as HRP [2,9,27]. Potential cofactors in mammalian cells were tested. Because glutathione peroxidase (GPx) is present in abundance in mammalian cells, GPx was tested to determine if it might participate in these kinds of reactions, either directly or by an intermediate. Figure 7 shows that GPx, unlike HRP, does not activate the probes by itself. However, the addition of H_2O_2 results in an increase in fluorescence of DHR123, and to a lesser extent DCHF, but not the other probes. To test if there is a glutathione intermediate that could oxidize the other probes, glutathione and H_2O_2 were added to the GPx solution (see Fig. 7). No increase in fluorescence was noted. The positive control, the addition of HRP, resulted in a rapid increase in fluorescence. These results indicate that GPx (a nonheme peroxidase) has little, if any, effect on these fluorescent probes.

Although extracellular catalase has been shown to decrease intracellular fluorescence when extracellular H_2O_2 is added to cells [4,8], we tested if catalase itself may interact directly with these probes. Surprisingly, catalase increased the fluorescence of all four probes and the addition of H_2O_2 further increased the fluorescence

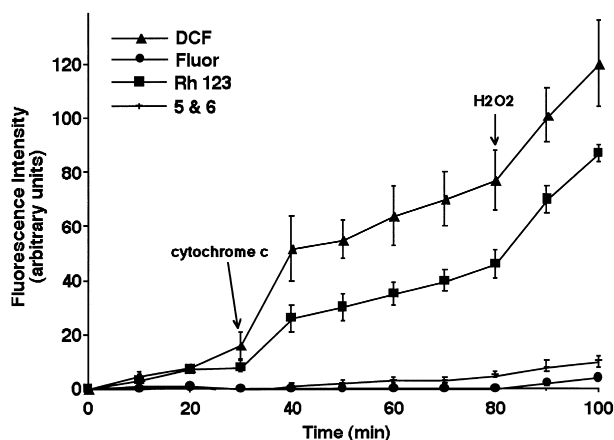


Fig. 10. Cytochrome c oxidizes dichlorodihydrofluorescein and dihydrorhodamine 123 and acts as a peroxide cofactor. After 30 min observation, 10 μM cytochrome c was added (arrow), then 100 nM H_2O_2 (arrow) ($n = 4$, $\pm$ SD).

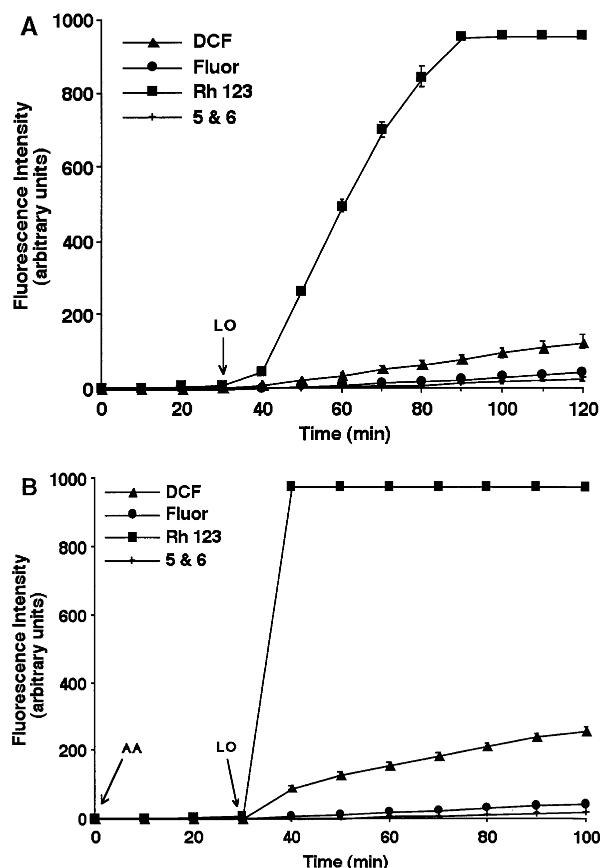


Fig. 11. 5-lipoxygenase plus arachidonic acid oxidizes DHR123 and DCHF. (A) At 30 min 4666 U/ml of lipoxygenase were added. (B) Arachidonic acid 150 μM was added to the buffer and fluorescence measurements started. At 30 min 4666 U/ml of lipoxygenase were added.

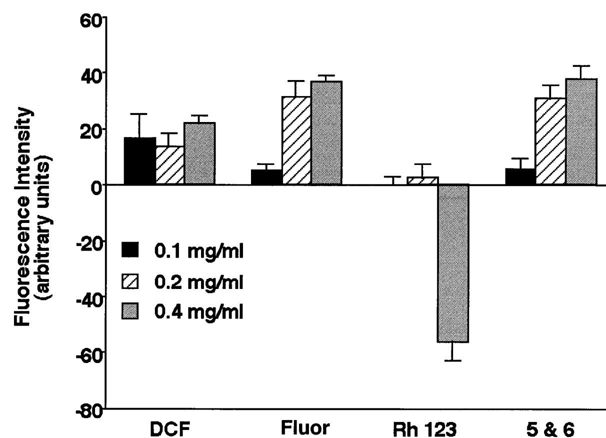


Fig. 12. Potassium superoxide addition results in oxidization of dihydrofluorescein and 5&6DH. KO_2 was added to each of the probes at the concentrations shown. After 5 min, a single fluorescence measurement was recorded. The negative Rh 123 fluorescence is due to reduction of baseline fluorescence of Rh 123 ($n = 4$, $\pm$ SD).

(Fig. 8A). This result indicates that catalase by itself may act as an intracellular factor able to oxidize these probes to fluorescence [26] and that catalase may act as a cofactor for the reaction with H_2O_2, perhaps due to peroxidase activity [28]. CuZn superoxide dismutase (Cu/Zn-SOD) can also oxidize the probes and also may act as a cofactor for the reaction with H_2O_2 (Fig. 8B). Similar results are observed with catalase and Cu/Zn-SOD together (Fig. 8C). These findings demonstrate that these two intracellular antioxidant enzymes may themselves oxidize the probes to fluorescence and that these enzymes can act as cofactors for the oxidation of these probes by H_2O_2.

Zhu previously reported that xanthine oxidase could oxidize DCHF to fluorescence [26]. These observations are expanded in Fig. 9, demonstrating that xanthine oxidase oxidizes all four probes, though it is most reactive toward DHR123. The addition of xanthine (50 μM) did not alter the rate of probe oxidation (data not shown), different than reports of hypoxanthine inhibiting the oxidation of DCHF by xanthine oxidase [26]. These results indicate that xanthine oxidase, by itself, may activate these probes to fluorescence.

Cytochrome *c* plus H_2O_2 is reported to oxidize DHR123 [9]. The four probes were tested with cytochrome c and H_2O_2 to determine the relative reactivity (Fig. 10). These results indicate that DHR123 and DCHF increase in fluorescence in the presence of cytochrome c. Addition of peroxide results in further increases in fluorescence, suggesting redox cycling (peroxidase activity) of H_2O_2 with cytochrome c and these two fluorescent probes. HFLUOR and 5&6DH were not reactive with cytochrome c alone and only weakly reactive with the addition of H_2O_2.

Intracellular lipid peroxides may be important markers of cell oxidant stress. To determine if these probes may detect lipid peroxides, the probes were exposed to 5-lipoxygenase without and with arachidonic acid in buffer solution (Fig. 11). Figure 11A shows that lipoxygenase by itself will oxidize DHR123, and to a minimal extent DCHF. Arachidonic acid oxidizes none of the probes (Fig. 11B), whereas the addition of lipoxygenase to the arachidonic acid results in rapid oxidation of DHR123 and less rapid oxidation of DCHF. HFLUOR and 5&6DH are minimally reactive under these conditions. The previous results with *tert*-butylhydroperoxide imply that the probes will not react with organic hydroperoxides in the absence of a cofactor, such as Fe^{2+}, or lipoxygenase.

Superoxide

Published reports indicate that the fluorescent probes DCHF and DHR123 are not oxidized by $O_2^{\bullet -}$ [8,9,26].

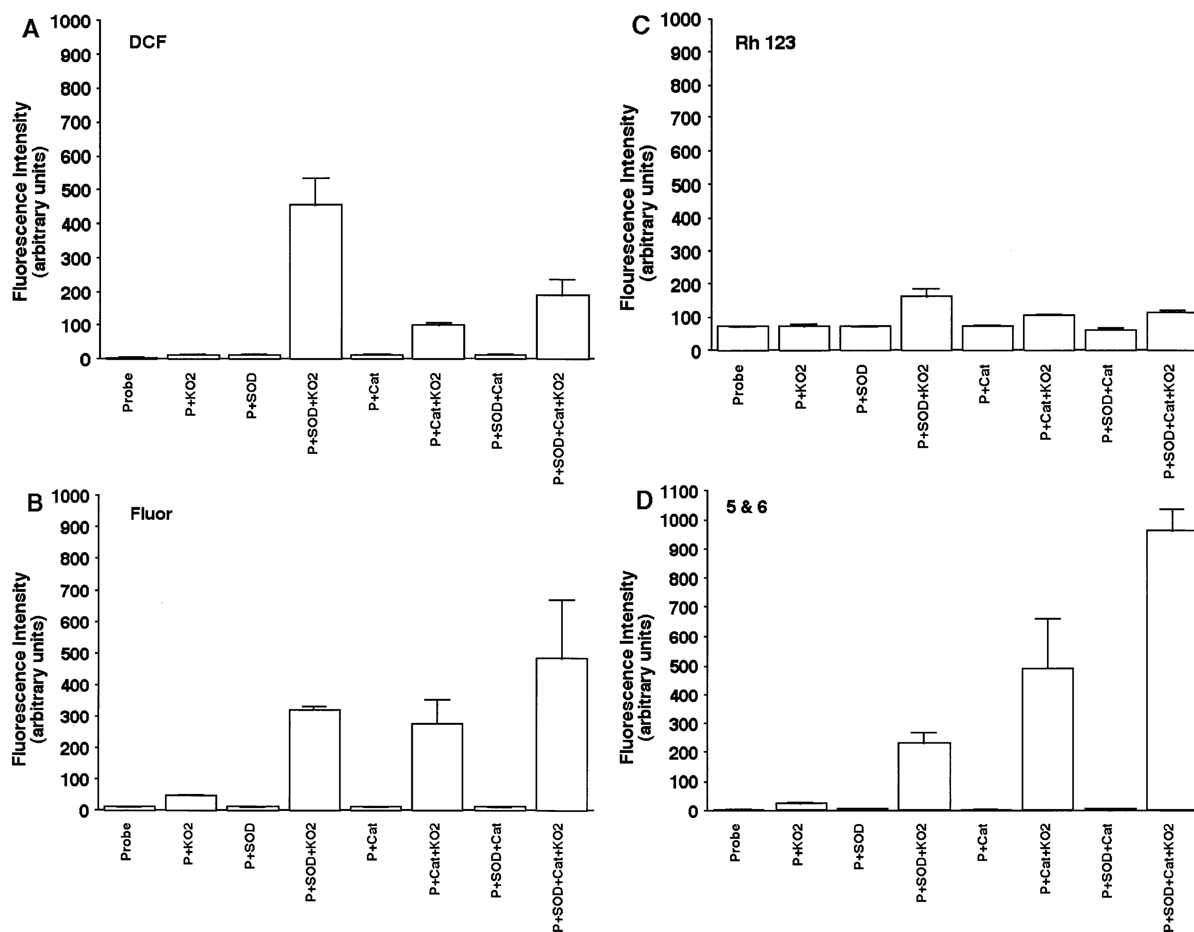


Fig. 13. Catalase and Cu/Zn-SOD are cofactors for the oxidation of fluorescent probes by potassium superoxide. In PBS buffer 5 μM fluorescent probe was exposed to buffer alone (Probe), probe plus 0.2 mg/ml KO_2 (P + KO2), probe plus 5 U/ml Cu/Zn-SOD (P + SOD), probe plus Cu/Zn-SOD plus KO_2 (P + SOD + KO2), probe plus 50 U/ml catalase (P + Cat), probe plus catalase plus KO_2 (P + Cat + KO2), probe plus catalase plus Cu/Zn-SOD (P + SOD + Cat), and probe plus Cu/Zn-SOD plus catalase plus KO_2 (P + SOD + Cat + KO_2). After 5 min, a single fluorescence measurement was recorded. (A) Oxidation of 2′,7′-dichlorodihydrofluorescein. (B) Oxidation of dihydrofluorescein. (C) Oxidation of dihydrorhodamine 123. (D) Oxidation of 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein (n = 4, ± SD).

To test this, the four probes were exposed to increasing concentrations of KO_2 (Fig. 12). KO_2 decreased the fluorescence of DHR123 (there is some basal oxidation of DHR123 in solution). KO_2 had little effect on DCHF, but modestly increased the fluorescence of HFLUOR and 5&6DH in a dose-dependent manner.

To expand these observations and to determine if $O_2^{\bullet -}$, in the presence of a cofactor, might oxidize the probes to fluorescence, KO_2 was added to each of the four probes in the presence of catalase and/or Cu/Zn-SOD (Fig. 13). In Fig. 13C DHR123 fluoresced little with KO_2 alone, but the cofactors Cu/Zn-SOD and/or catalase increased fluorescence of DHR123 slightly. However, the other three probes fluoresced brightly with Cu/Zn-SOD and/or catalase after adding KO_2. HFLUOR and 5&6DH were the most fluorescent, particularly in the presence of Cu/Zn-SOD plus catalase. These results indicate that KO_2, in the presence of Cu/Zn-SOD and/or catalase, oxidizes the probes to fluorescence. These findings imply that intracellular $O_2^{\bullet -}$ formation could oxidize three of the probes to fluorescence, HFLUOR and 5&6DH being the most reactive. In the absence of a cofactor, fluorescence is low, suggesting that $O_2^{\bullet -}$ by itself is inefficient in generating fluorescence from these probes.

Nitric oxide and peroxynitrite

Recent reports indicate that fluorescent probes may react with peroxynitrite ($ONOO^-$) and $NO^\bullet$ [29,30]. Figure 14 demonstrates the reactivity of the probes with $ONOO^-$. It shows that DCHF is the most reactive with peroxynitrite. DHR123 increased in fluorescence after the first addition of $ONOO^-$, but then became unreactive

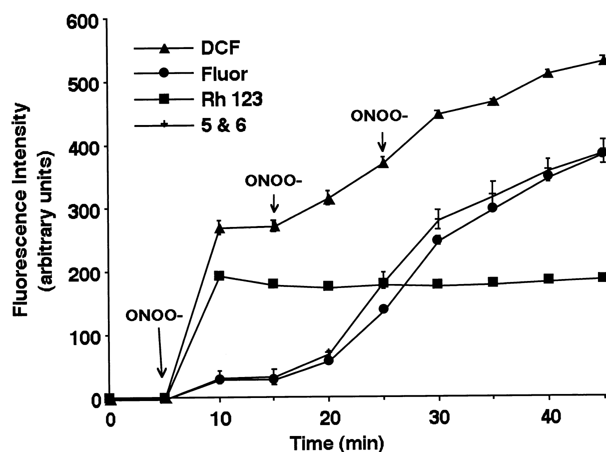


Fig. 14. Peroxynitrite oxidizes the fluorescent probes. After 5 min observation, three additions of 400 nM peroxynitrite (arrows) were added to 5 μM fluorescent probe at 10 min intervals (n = 4, ± SD).

to additional $ONOO^-$. HFLUOR and 5&6DH reacted with $ONOO^-$ to a similar degree, but were less reactive than DCHF. In Fig. 15, the four probes were exposed to $NO^•$. DCHF was the only probe that fluoresced upon addition of $NO^•$. The relative reactivity of DCHF with $ONOO^-$ was much higher than the reactivity with $NO^•$ (Figs. 14 and 15).

Molar fluorescence

These results examine specific reactions that may oxidize the probes. Results are expressed as fluorescent intensity. This is a useful approach to examine specific reactions that may influence the brightness of intracellular fluorescence observed under the confocal microscope. However, this approach does not account for potential differences in the molar fluorescence of each probe. Table 1 shows the relative fluorescence of each probe at the excitation and emission wavelengths of our confocal microscope and at the optimum excitation and emission wavelengths of each probe. These results indicate that that fluorescein has the highest molar fluorescence of the four probes examined.

Table 1. Relative Molar Fluorescence of Oxidized Probes

Probe	Exλ/ Emλ nm	Fluorescence	Relative to DCF	Exλ/Emλ nm	Fluorescence
DCF	488/521	410	1.00	502/521	616
Fluor	488/521	592	1.44	490/512	730
Rh 123	488/521	458	1.12	499/527	579
5 & 6	488/521	307	0.75	504/523	501

Reagent grade oxidized probes were used to determine the relative molar fluorescence of each probe. 0.5 μM (final) of oxidized fluorescent probe (100 μM working stock in methanol) was added to 50 mM potassium-phosphate buffer, pH 9.0, at room temperature. The concentrations were confirmed by the molar extinction coefficients supplied by the manufacturer. The first set of Exλ/Emλ values represent the excitation and emission wavelengths of the confocal microscope. "Relative to DCF" denotes the fluorescence at Exλ 488/Emλ 521 compared with DCF. The second set of Exλ/Emλ values represent the optimum excitation and emission values for the specific probes. These second Exλ/Emλ values were obtained by scanning and differ slightly from the published values shown in Fig. 1.

Cell uptake of fluorescent probes

Another mechanism to account for variability of cellular fluorescence by different probes may be different uptake of the fluorescent probes. To test this, cells were loaded for 15, 30, or 60 min with DCHF-DA or HFLUOR-DA. Cells were then lysed and intracellular fluorescence measured. The nonfluorescent reduced intracellular probes were then oxidized with HRP and fluorescence measured again, a modification of the technique previously described by Royal and Ischiropoulos [9]. Results, shown in Table 2, demonstrate that most of the intracellular probe is not oxidized by the cell's basal metabolism. After treatment with HRP, the fluorescence from cells treated with HFLUOR-DA is about three times higher than DCHF-DA–treated cells. Both probes demonstrate substantial increases in fluorescence after HRP treatment. This implies the presence of a large amount of reduced probe in the cell interior that may be available for oxidation by an intracellular oxidant stress. The increased amount of intracellular HFLUOR available for oxidation may be another factor accounting for the higher fluorescence, as shown in Figs. 3 and 4.

DISCUSSION

This work illustrates that the oldest fluorescent probe, dihydrofluorescein [19], as the diacetate [18], is a useful probe for cell-based investigations. We compared the most popular probes with dihydrofluorescein diacetate, expanding observations by others, and presenting previously unreported information. For example, the ability of catalase and Cu/Zn-SOD by themselves to increase fluorescence of the probes is a new observation. In addition, the activity of catalase and Cu/Zn-SOD as cofactors for probe oxidation by H_2O_2 is also new. During this investigation several previously unobserved reactions with KO_2 were also identified. It was previously reported that these probes are not oxidized by $O_2^{•-}$ [8,9,26]. However, the results reported here demonstrate that HFLUOR and 5&6DH are oxidized by the addition of KO_2. The specific species of reduced oxygen that results in oxidation of HFLUOR and 5&6DH when KO_2 is added is unknown. Although reaction with H_2O_2 generated by KO_2 is a possibility [8,31], these probes react slowly with

Table 2. Cellular Uptake and Oxidation of DCHF-DA and HFLUOR-DA

	Buffer Fluorescence		Intracellular Fluorescence		HRP Fluorescence	
Loading	DCF	Fluor	DCF	Fluor	DCF	Fluor
15 min	27 ± 1	14 ± 0	115 ± 8	116 ± 5	1221 ± 153	3404 ± 141
30 min	65 ± 5	38 ± 4	126 ± 6	124 ± 7	1940 ± 62	5560 ± 113
60 min	140 ± 5	90 ± 3	120 ± 6	121 ± 16	2028 ± 40	6353 ± 342

Endothelial cells on 24 well plates were loaded with 20 μM DCHF-DA or HFLUOR-DA for 15, 30, or 60 min as shown. After loading, the cells were washed, and the extracellular buffer fluorescence (probe loading buffer plus washes) was measured at Exλ 488/Emλ 521 (buffer fluorescence) on the Perkin-Elmer LS-5OB luminescence spectrometer. The cells were then lysed with 0.1% SDS and the intracellular fluorescence measured (intracellular fluorescence). The cell lysates were oxidized overnight with 30 U/ml HRP (HRP fluorescence). For valid comparisons, the values for HRP fluorescence were mathematically corrected for dilution and smaller Em/Ex slit widths (n = 4, ± SD).

H_2O_2 (Fig. 5B). This implies that a species, generated by $O_2^{\bullet -}$, oxidizes HFLUOR and 5&6DH. These studies also demonstrate that the enzymes Cu/Zn-SOD and/or catalase can markedly increase the oxidation of DCHF, HFLUOR, and 5&6DH by KO_2. In fact, 5&6DH, which tended to be the least reactive probe in other reactions, was the most readily oxidized in the presence of KO_2 with catalase and Cu/Zn-SOD. Finally, these results demonstrate that oxidized rhodamine is reduced by KO_2 (Fig. 5B), even though DHR123 is the probe most readily oxidized by H_2O_2 in our previous experiments.

The experimental results presented in Fig. 2 demonstrate the potential utility of HFLUOR in imaging sites of cellular oxidant generation. There is a good signal-to-noise ratio, allowing HFLUOR to readily demonstrate linear fluorescence in the reoxygenated cells, whereas the control cells show little fluorescence. The "brightness" observed in Fig. 3 using this probe demonstrates its relative superiority to the other probes under carefully controlled conditions. These results were confirmed using a fluorescence plate reader, Fig. 4. These results

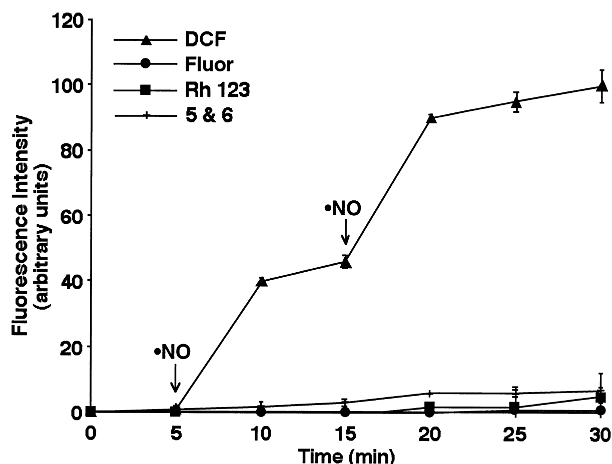


Fig. 15. Nitric oxide addition results in the oxidation of 2′,7′-dichlorodihydrofluorescein. After 4 min observation, two aliquots of 20 μM (final) nitric oxide (arrows) were added to 5 μM fluorescent probe (n = 4, ± SD).

caused us to test the hypothesis that HFLUOR was brighter than the other probes because of enhanced reaction rate toward specific oxidants. However, the results in subsequent experiments did not confirm the hypothesis. Rather, we discovered that HFLUOR was often less reactive toward oxidants compared to the probes DHR123 and DCHF.

Although this work did not demonstrate the specific mechanism by which HFLUOR generates brighter confocal images, there are several potential mechanisms. One possibility is that there are additional intracellular reactions not studied here. Another possibility is that this probe localizes to regions of intracellular oxidant formation better than the other probes. Another possibility is that HFLUOR may be brighter due to higher molar fluorescence (see Table 1). Finally, HFLUOR-DA may be taken up or retained by the cell better than the other probes as indicated by Table 2.

Though some of the reactions reported here are previously described by others, this work expands much of the previous work by comparing the three most popular probes to each other and introducing the oldest fluorescent reagent [19], dihydrofluorescein, as a probe that may be useful for cell-based studies. These comparisons may assist investigators in choosing a specific probe for their investigations.

We have demonstrated that there are numerous reactions that may oxidize dihydrofluorescein and the other three probes. Based on these results, and previous reports, it is not proper to assume that these oxidant-sensing probes react only with H_2O_2 [6,8,20], but rather act as detectors of a broad range of intracellular oxidizing reactions.

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ABBREVIATIONS

DCHF—2′,7′-dichlorodihydrofluorescein
DCHF-DA—2′,7′-dichlorodihydrofluorescein diacetate
DHR123—dihydrorhodamine 123
5&6DH—5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein
5&6DH-DA—5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate
GPx—glutathione peroxidase
HFLUOR—dihdydrofluorescein
HFLUOR-DA—dihydrofluorescein diacetate
HRP—horseradish peroxidase
Cu/Zn-SOD—copper/zinc superoxide dismutase
DCF—2′,7′-dichlorofluorescein
Fluor—fluorescein
Rh 123—rhodamine 123
5 & 6—5(and 6)-carboxy-2′,7′-dichlorofluorescein