Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY$^{581/591}$

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Abstract A ratio-fluorescence assay was developed for on-line localization and quantification of lipid oxidation in living cells. The assay explores the oxidative sensitivity of C11-BODIPY$^{581/591}$. Upon oxidation, the fluorescence of this fluorophore shifts from red to green. The probe incorporates readily into cellular membranes and is about twice as sensitive to oxidation as arachidonic acid. Using confocal microscopy, the cumene hydroperoxide-induced oxidation of C11-BODIPY$^{581/591}$ was visualized at the sub-cellular level in rat-1 fibroblasts. Preloading of the cells with tocopherol retarded this oxidation. The data demonstrate that C11-BODIPY$^{581/591}$ is a valuable tool to quantify lipid oxidation and anti-oxidant efficacy in single cells.

Key words: BODIPY; Lipid oxidation; Oxygen radical; Ratio-fluorescence microscopy; Tocopherol

1. Introduction

Polyunsaturated fatty acids (PUFAs) have a broad range of cell biological functions affecting membrane integrity, transmembrane transport [1,2], signal transduction and gene transcription [3]. PUFAs are opportune targets for reactive oxygen species (ROS) which are produced both enzymatically (e.g. oxidases) and in processes such as respiration. A wealth of information about PUFA oxidation has been provided by assays that are based on the detection of secondary reaction products (e.g. aldehydes, isoprostanes) [4,5]. These methods, however, are relatively elaborate and do not provide (sub)-cellular resolution. Complementary to these methods, oxidation-sensitive fluorophores have been applied in model systems and cultured cells. The most commonly used fluorophore is the fatty acid analogue *cis*-parinaric acid (*cis*-PnA) [6–8]. However, its fluorescence in the UV range hinders direct measurement of lipid peroxidation in intact cells. Moreover, its extensive unsaturation makes this probe much more susceptible to oxidation than biologically relevant PUFAs such as arachidonic acid [8]. Other probes used to monitor oxidation processes in cellular membranes are undecylamine fluorescein and hexadecanoyl BODIPY-FL [9,10]. Since only the non-oxidized form of these fluorophores can be detected, inaccuracies as a result of variations in cellular uptake and of compartmentalization cannot be corrected. In other fields such as pH, Ca^{2+} and ion imaging, such inaccuracies have been circumvented by the use of ratio-fluorescence dyes. Here, we show that the fluorophore C11-BODIPY$^{581/591}$ is very suitable for ratio-imaging of oxidation processes in membranes of living cells. While this study was in progress it was reported that C11-BODIPY$^{581/591}$ is susceptible to oxidation thereby losing its bright red fluorescence [11].

2. Materials and methods

2.1. Chemicals

2,2′-Azobis(2-amidinopropane hydrochloride) was obtained from Polysciences (Warrington, PA, USA). All fluorophores used were obtained from Molecular Probes (Junction City, OR, USA). Egg phosphatidylcholine (egg PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals used were obtained from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Cell culture

Myocardial cells and rat-1 fibroblasts were cultured as described previously [7,12].

2.3. Preparation of vesicles

Small unilamellar vesicles of egg PC were made through ethanolic injection [13]. The C11-BODIPY$^{581/591}$ concentration of the stock solutions was determined by measuring the absorption at 581 nm on a Hitachi U-2000 dual beam spectrophotometer using a molar extinction coefficient of 139 444 l mol^{-1} cm^{-1} [14].

2.4. Labeling of cells with C11-BODIPY$^{581/591}$ and with cis-PnA

Cells were incubated for 30 min at 37°C with C11-BODIPY$^{581/591}$ (1 μM) in growth medium. Prior to use, myocardial cells were rinsed with buffer W^+ (133 mM NaCl, 5 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 1 mM $CaCl_2{\cdot}2H_2O$, 10 mM Tris-HCl, 5 mM glucose, $pH_{37°C}$ 7.4). Rat-1 fibroblasts were rinsed with enriched phosphate buffered saline (PBS^+, 137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and 5 mM glucose, pH 7.4). The labeling of cells with *cis*-PnA was performed as described previously [8].

2.5. Induction of oxidation

In the imaging experiments, oxidation of C11-BODIPY$^{581/591}$ was induced by adding cumene hydroperoxide (CumOOH) with or without hemin in PBS^+. To compare the relative sensitivity of C11-BODIPY$^{581/591}$ and of arachidonic acid, oxidation was induced by incubating the cells with 50 μM CumOOH at 37°C. Aliquots were taken from the buffer to measure lactate dehydrogenase (LDH) release [15].

2.6. Extraction of lipids

Cells were extracted for 60 min at ambient temperature with 2 ml isopropanol (0.05% butylated hydroxytoluene) as described before [15]. Total phospholipid content of cells was determined spectrophotometrically according to Rouser et al. [16].

2.7. Confocal laser scanning microscopy

For fluorescence microscopy, cells were plated at least 24 h before

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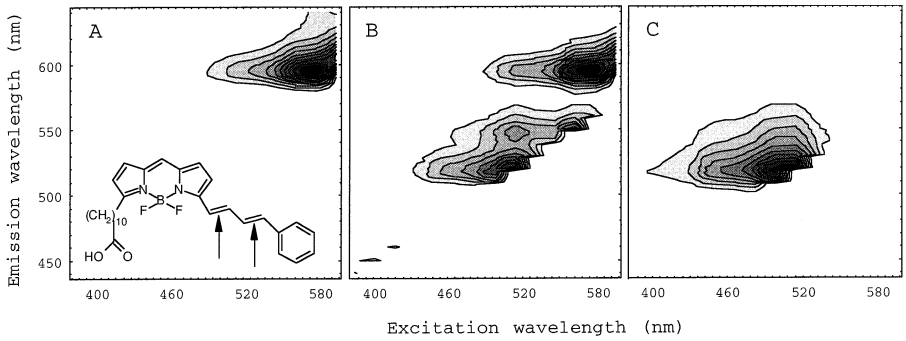

Fig. 1. Contour plots of the fluorescence of C11-BODIPY$^{581/591}$ (0.1 mol%) in egg PC vesicles (100 μM egg PC); non-oxidized (A), and oxidized after 45 min (B) and 2 h (C) of exposure to 80 μM CumOOH and 80 nM hemin. The chemical structure of C11-BODIPY$^{581/591}$ is presented in the inset of A. The arrows indicate the diene bond likely to be oxidized by radicals.

use on coverslips (diameter 24 mm). Samples were placed in the temperature (37°C)-controlled coverslip holder of the microscope. Images were taken with a Leica TCSNT confocal laser scanning system on an inverted microscope DMIRBE (Leica Microsystems, Heidelberg, Germany) with an argon-krypton laser as excitation source. The green and red fluorescence of C11-BODIPY$^{581/591}$ was acquired simultaneously using double wavelength excitation (laserlines 488 and 568 nm) and detection (emission bandpass filters 530/30 and 590/30). When the probe to lipid molar ratio exceeds 1:20, BODIPY fluorophores are known to form dimers between excited and non-excited BODIPY monomers (so-called excimers) which due to their red-shifted emission wavelength could interfere in the ROS measurements. The formation of excimers was routinely excluded by measuring the fluorescence emission at 600/30 nm while exciting at 488 nm. The excimer fluorescence at these settings was negligibly low.

2.8. Fluorometry

Fluorescence measurements were carried out at ambient temperature using a Quantum Master spectrofluorometer (PTI, Surbiton, Surrey, UK) [8]. Fluorescence of C11-BODIPY$^{581/591}$ was measured by simultaneous acquisition of the green (484/510 nm) and red signals (581/610 nm). A Tecan multiwell spectrofluorometer (Tecan, UK) was used to measure the oxidation of C11-BODIPY$^{581/591}$ in vesicles in 96 well plates. The red fluorescence was selectively detected using the excitation and emission bandpass filters of 590/15 and 635/20 nm, respectively.

2.9. Partition coefficient

Lipophilicity of C11-BODIPY$^{581/591}$ was determined by measuring the partition between the medium and erythrocyte ghosts at 37°C. The apparent partition coefficient was calculated relative to the bilayer volume using data reported by Scheufler and Peters [17].

2.10. Fatty acid analysis

The fatty acid composition of the phospholipid extracts was determined by capillary gas chromatography using a Perkin Elmer 8500 gas chromatograph as described before [8].

3. Results

3.1. Spectral shift of BODIPY$^{581/591}$ fluorescence upon oxidation

The fluorophore C11-BODIPY$^{581/591}$ is composed of a boron dipyromethene difluoride core that is extended with a phenyl moiety (see inset, Fig. 1A). The contour plot of the fluorescence of C11-BODIPY$^{581/591}$ in egg PC vesicles is presented in Fig. 1A. The wavelengths of maximal excitation and emission corresponded to 581 and 591 nm, respectively. Addition of CumOOH/hemin, as an initiator of lipid oxidation, shifts the excitation and emission spectra to shorter wavelengths corresponding to green fluorescence (Fig. 1B,C, peak excitation 500 nm, emission 510 nm). Since both forms of C11-BODIPY$^{581/591}$ are spectrally well separated, this property can be used to quantify the fraction of oxidized and non-oxidized C11-BODIPY$^{581/591}$ simultaneously at any time point.

To assess the chemical basis of this spectral shift, several other substituted red-fluorescent BODIPY derivatives were tested (C12-BODIPY$^{558/568}$, BODIPY-TR cadaverin) which have an extended conjugated bond system, but lack the diene bond typical of C11-BODIPY$^{581/591}$. Addition of CumOOH/hemin to these derivatives had no effect on the fluorescent properties. These observations suggest that in particular the oxidation of the diene bond is responsible for the fluorescence shift of C11-BODIPY$^{581/591}$. After oxidation of this bond, the remaining conjugation of C11-BODIPY$^{581/591}$ is identical to that of the unsubstituted boron dipyromethene difluoride dye (BODIPY$^{500/510}$) exhibiting green fluorescence.

3.2. Oxidant specificity of C11-BODIPY$^{581/591}$

To determine the reactivity of C11-BODIPY$^{581/591}$, the probe was dispersed in egg PC vesicles and exposed to various ROS. Fig. 2A illustrates that the sequential addition of CumOOH and hemin leads to a decay of the red fluorescence and a concomitant appearance of the green fluorescence. From the decay curves the initial oxidation rates (IPR) were determined. As shown in Fig. 2B, C11-BODIPY$^{581/591}$ is oxidized only when CumOOH and hemin are present. The rate of C11-BODIPY$^{581/591}$ oxidation correlates directly with the CumOOH concentration, up to 0.2 mM. At higher CumOOH concentrations, the oxidation rate levels off due to hemin (80 nM) becoming limiting.

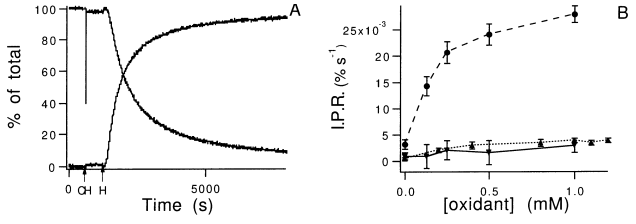

Fig. 2. A: Time curves of green (ex/em 490/510 nm) and red fluorescence (ex/em 580/600 nm) of C11-BODIPY$^{581/591}$ in egg PC vesicles prior to and after sequential addition of CumOOH (CH, 0.5 mM) and hemin (H, 80 nM). B: Initial peroxidation rates of C11-BODIPY$^{581/591}$ in egg PC vesicles exposed to various concentrations of either CumOOH in the presence (●) and absence (▼) of 80 nM hemin or SIN-1 (▲).

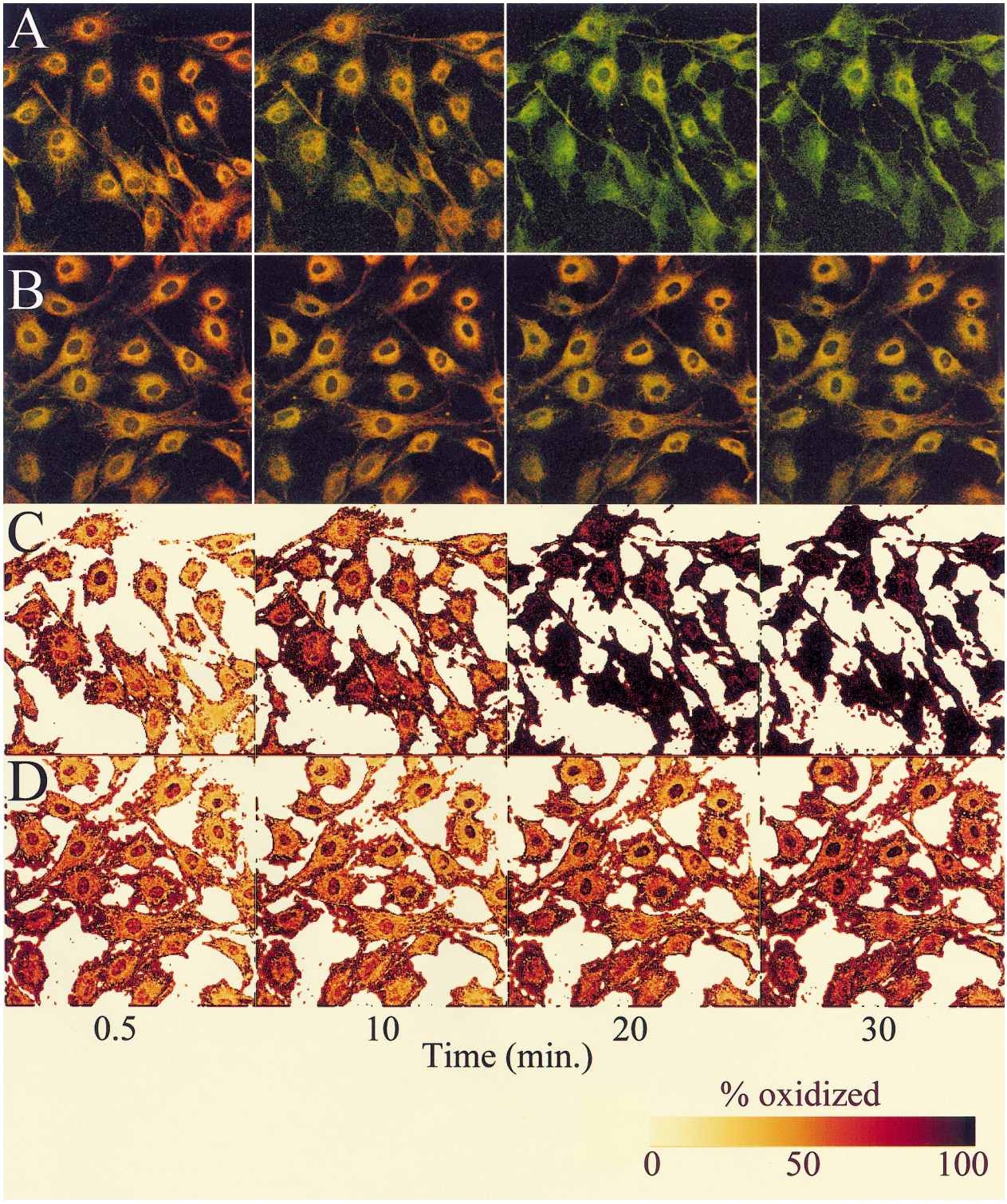

Fig. 3. Serial images of rat-1 fibroblasts loaded with C11-BODIPY$^{581/591}$ and exposed to 80 μM CumOOH and 80 nM hemin (A). In B, the cells were pre-incubated with α- and γ-tocopherol for 16 h prior to exposure to the oxidants. In C and D, the fraction of oxidized C11-BODIPY$^{581/591}$ is presented. To obtain this fraction the green fluorescence is divided by the total fluorescence (green+red).

C11-BODIPY$^{581/591}$ is also efficiently oxidized by other hydroxy-, peroxy- and oxy-radical generating systems such as hydrogen peroxide/Fe^{2+} and 2,2′-azobis(2-amidinopropane hydrochloride) (data not shown). However, this probe is relatively insensitive to 3-morpholinosydnonimine hydrochloride (SIN-1), which generates nitric oxide and superoxide [18].

3.3. Oxidation of C11-BODIPY$^{581/591}$ in rat-1 fibroblasts

The lipophilicity of the probe ,as determined by its partitioning into erythrocyte ghost membranes, yielded a partition coefficient of 27 254 (±84). Administration of C11-BODIPY$^{581/591}$ dissolved in fetal calf serum to the growth medium of rat-1 fibroblasts leads to a rapid incorporation into cellular

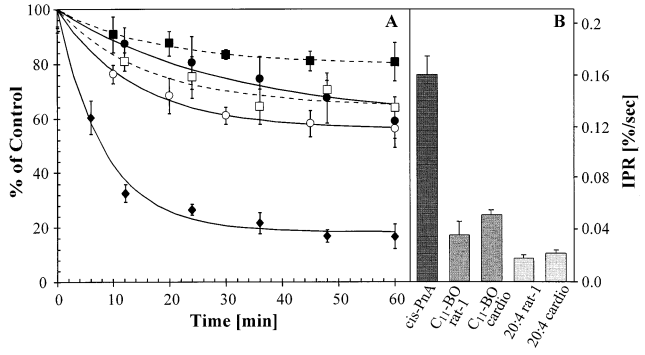

Fig. 4. Depletion of arachidonic acid (■, ●) and C11-BODIPY$^{581/591}$ (□, ○) in rat-1 fibroblasts (dashed curves) and in cardiomyocytes (solid curves) exposed to 50 μM CumOOH. In addition, the decay of *cis*-PnA in cardiomyocytes is given (♦). Analysis of the curves yielded the initial peroxidation rates (IPR) as given in B [8].

membranes. After 30 min of labeling, the ratio of C11-BODIPY$^{581/591}$ to cellular phospholipid is 1:13 047 ± 734 which ensures a minimal perturbation of the membranes and the absence of spectroscopic artifacts such as self-quenching or excimer formation [10,14]. Indeed, excimers of oxidized C11-BODIPY$^{581/591}$ were not observed with this labeling procedure (see Section 2). To investigate whether the probe incorporated into rat-1 fibroblasts is susceptible to oxidation, we exposed the cells to CumOOH and hemin. The C11-BODIPY$^{581/591}$ fluorescence after 0.5–30 min of exposure to this radical generating system is presented in Fig. 3A. From the color change we conclude that within 30 min oxidation of the probe is nearly complete. From the green and red fluorescence intensities the fraction of oxidized C11-BODIPY$^{581/591}$ was calculated in each pixel (Fig. 3C). As is clearly seen from the first panel (0.5 min), oxidation of the probe is particularly pronounced (about 50%) at the cell periphery. With time the probe becomes gradually oxidized throughout the cell. Oxidation of C11-BODIPY$^{581/591}$ inside the cell may be caused either by secondary radicals derived from chain reactions at the plasma membrane, or by radicals initiated by CumOOH reacting with intracellular transition metals. In addition, it cannot be excluded that C11-BODIPY$^{581/591}$ oxidized at the cell periphery redistributes throughout the cell. In Fig. 3B,D it is shown that oxidation of C11-BODIPY$^{581/591}$ is strongly attenuated when rat-1 fibroblasts were pretreated with a mixture of α- and γ-tocopherol at physiological concentrations (25 and 1.25 μM, respectively). Since the cells were washed vigorously before exposure to CumOOH/hemin, the observed protection results from tocopherol inserted into the cellular membranes. By measuring LDH release it was confirmed that upon treatment with tocopherol, the plasma membrane integrity was strongly preserved (9.9% (± 2.5) of LDH release as compared with a release of 79.6% (± 7) in control cells after 2 h).

To investigate to what extent the fluorescence shift of C11-BODIPY$^{581/591}$ in cells relates to the oxidation of arachidonic acid, we compared their sensitivity to CumOOH in rat-1 fibroblasts and in cardiomyocytes. After different times of exposure, lipids were extracted and the arachidonic acid content estimated by gas chromatography. From the same extracts, the levels of non-oxidized C11-BODIPY$^{581/591}$ and of *cis*-PnA were determined spectroscopically (Fig. 4A). In both cell systems, the initial peroxidation rate of C11-BODIPY$^{581/591}$ is about twice that observed for arachidonic acid (Fig. 4B). The oxidative sensitivity of *cis*-PnA is much higher.

4. Discussion

Oxidative damage to PUFA is increasingly being implicated as a primary cause of various diseases and ageing [19–23]. In order to adequately investigate the radical physiology and the efficacy of antioxidants, there is great demand for simple and sensitive methods that provide spatial and temporal information about oxidative processes in cultured cells and tissues. An important limitation of the common oxidation-sensitive dyes is that that either the non-oxidized form (in the case of *cis*-PnA, fluorescein, BODIPY-FL) or the oxidized form (in the case of resorufin derivatives) is detected. As a consequence, essential corrections for dye uptake and compartmentalization cannot be made. We show that the unique properties of C11-BODIPY$^{581/591}$ facilitate 'ratio-fluorescence' imaging of ROS activities in membranes of living cells. As long as the conjugated system of this fluorophore is intact, the observed fluorescence is red (591 nm). Upon oxidation, the red fluorescence of C11-BODIPY$^{581/591}$ shifts to green (510 nm). From the images taken at different wavelengths, the fraction of oxidized probe can be calculated at each time point in each pixel of a digital microscopic image. This fraction is independent of heterogeneous probe distribution and/or fluorescence quenching. We conclude that C11-BODIPY$^{581/591}$ is very suited for ratio-fluorescence imaging of reactive oxygen in membranes of living cells.

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