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Minireview

Molecules and Cells

Methods for Detection and Measurement of Hydrogen Peroxide Inside and Outside of Cells

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Hydrogen peroxide (H_2O_2) is an incompletely reduced metabolite of oxygen that has a diverse array of physiological and pathological effects within living cells depending on the extent, timing, and location of its production. Characterization of the cellular functions of H_2O_2 requires measurement of its concentration selectively in the presence of other oxygen metabolites and with spatial and temporal fidelity in live cells. For the measurement of H_2O_2 in biological fluids, several sensitive methods based on horseradish peroxidase and artificial substrates (such as Amplex Red and 3,5,3′5′-tetramethylbenzidine) or on ferrous oxidation in the presence of xylenol orange (FOX) have been developed. For measurement of intracellular H_2O_2, methods based on dihydro compounds such as 2′,7′-dichlorodihydrofluorescein that fluoresce on oxidation are used widely because of their sensitivity and simplicity. However, such probes react with a variety of cellular oxidants including nitric oxide, peroxynitrite, and hypochloride in addition to H_2O_2. Deprotection reaction-based probes (PG1 and PC1) that fluoresce on H_2O_2-specific removal of a boronate group rather than on nonspecific oxidation have recently been developed for selective measurement of H_2O_2 in cells. Furthermore, a new class of organelle-targetable fluorescent probes has been devised by joining PG1 to a substrate of SNAP-tag. Given that SNAP-tag can be genetically targeted to various subcellular organelles, localized accumulation of H_2O_2 can be monitored with the use of SNAP-tag bioconjugation chemistry. However, given that both dihydro- and deprotection-based probes react irreversibly with H_2O_2, they cannot be used to monitor transient changes in H_2O_2 concentration. This drawback has been overcome with the development of redox-sensitive green fluorescent protein (roGFP) probes, which are prepared by the introduction of two redox-sensitive cysteine residues into green fluorescent protein; the oxidation of these residues to form a disulfide results in a conformational change of the protein and altered fluorogenic properties. Such genetically encoded probes react reversibly with H_2O_2 and can be targeted to various compartments of the cell, but they are not selective for H_2O_2 because disulfide formation in roGFP is promoted by various cellular oxidants. A new type of H_2O_2-selective, genetically encoded, and reversible fluorescent probe, named HyPer, was recently prepared by insertion of a circularly permuted yellow fluorescent protein (cpYFP) into the bacterial peroxide sensor protein OxyR.

INTRODUCTION

Hydrogen peroxide (H_2O_2), a product of incomplete reduction of O_2, is generated as a by-product of a wide range of biological processes (D'Autreaux and Toledano, 2007; Giorgio et al., 2007; Rhee, 2006). For example, one-electron reduction of O_2 that occurs as a result of electron leakage from mitochondria or the oxygenation of organic molecules by cytochrome P450 enzymes initially generates the superoxide anion ($O_2^{-\bullet}$), which is then spontaneously or enzymatically (via the action of superoxide dismutase) converted to H_2O_2. Hydrogen peroxide is reduced further to the hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction in the presence of Cu^{2+} or Fe^{2+} ions. Hydrogen peroxide, the superoxide anion, and the hydroxyl radical are collectively known as reactive oxygen species (ROS). In general, ROS are toxic to cells because of their propensity to cause macromolecular damage. Although H_2O_2 is a mild oxidant, being the least reactive of ROS, all aerobic cells are equipped with various H_2O_2-eliminating enzymes because H_2O_2 is readily converted to the highly reactive hydroxyl radical via the Fenton reaction.

Despite its toxicity, a large body of experimental data indicates that H_2O_2 is also produced transiently in response to activation of various cell surface receptors and plays a key role as an intracellular messenger in mammalian cells (D'Autreaux and Toledano, 2007; Giorgio et al., 2007; Rhee, 2006). It acts on downstream target proteins within signal transduction cascades to modulate their functions through reversible oxidation of selective cysteine residues (D'Autreaux and Toledano, 2007; Giorgio et al., 2007; Janssen-Heininger et al., 2008; Rhee, 2006). NADPH oxidases and lipoxygenases are largely responsible for receptor-dependent H_2O_2 production. Various

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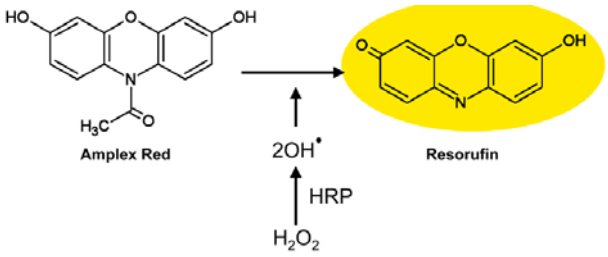

Fig. 1. Detection of H_2O_2 with Amplex Red. In the presence of HRP, H_2O_2 reacts stoichiometrically with Amplex Red to generate the red-fluorescent oxidation product, resorufin.

isoforms of these enzymes are activated at or in discrete subcellular compartments, including lipid rafts, endosomes, the endoplasmic reticulum, and the nucleus (Oakley et al., 2009; Ushio-Fukai, 2009). A recent study indicates that localized production and accumulation of H_2O_2 are critical for its signaling function (Woo et al., 2010). Hydrogen peroxide is also produced extracellularly by NADPH oxidase 2 as a means of bacterial killing by neutrophils. Given that membranes are highly permeable to H_2O_2, H_2O_2 produced in one compartment can diffuse into other compartments, and H_2O_2 is also known to mediate cell-to-cell communication.

Despite the importance of H_2O_2 to cellular activities, the molecular mechanisms of its production, accumulation, function, and degradation remain insufficiently understood. The acquisition of such knowledge is hampered in part by the difficulty of quantifying and tracking the small, diffusible H_2O_2 molecules with spatial and temporal fidelity in live cells. Although sensitive and selective methods are available for measurement of H_2O_2 outside of cells, it has proved difficult to devise a probe for the intracellular detection of H_2O_2 selectively over other ROS and reactive nitrogen species (nitric oxide and peroxynitrite) that are often generated together with H_2O_2. Furthermore, such a probe has to be cell permeable but remain trapped inside the cell after its reaction with H_2O_2. It is even more challenging to produce a probe that undergoes reversible reaction with H_2O_2 for real-time measurements or that can be delivered to different subcellular compartments for local detection. However, over the last 5 years or so, substantial progress has been made to meet these challenges.

In this review, we describe the various H_2O_2 detection methods developed to date and discuss their strengths and weaknesses. The background and general experimental procedures for each method are described, but these procedures will need to be modified depending on the specific experimental design and with full knowledge of the basic principles of the method and the instrumentation required.

Horseradish peroxidase-based fluorometric assay with Amplex Red

Background

Hydrogen peroxide reacts with Amplex® Red [*N*-acetyl-3,7-dihydroxyphenoxazine (Molecular Probes-Invitrogen, USA)] at a stoichiometry of 1:1 in a reaction catalyzed by horseradish peroxidase (HRP) to generate the highly fluorescent product resorufin. HRP catalyzes the decomposition of H_2O_2 to the hydroxyl radical, which is then reduced to water as a result of irreversible chemical oxidation of Amplex Red, a colorless and nonfluorescent derivative of resorufin, thereby generating fluorescent resorufin (Fig. 1). Resorufin exhibits a maximum of

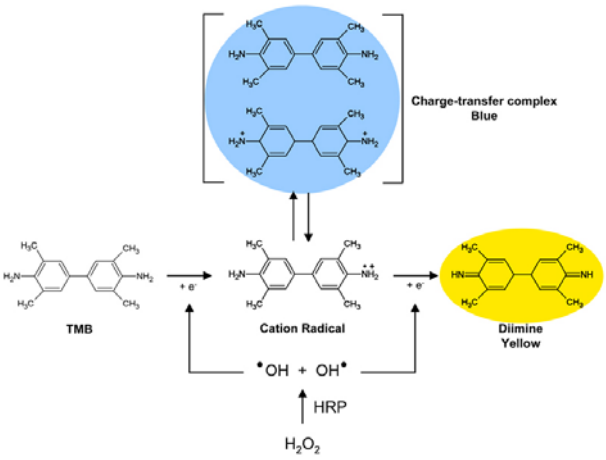

Fig. 2. Chemical structure of TMB and its oxidation products

fluorescence emission at a wavelength of 587 nm and maximum excitation at 563 nm, and its extinction coefficient (ε) is ~54,000 M^{-1} cm^{-1} (Zhou et al., 1997). The assay based on this reaction is highly sensitive, allowing measurement of H_2O_2 at concentrations as low as 50 nM in a 96-well fluorescence microplate format (Lyon and Stevenson, 2006; Zhou et al., 1997).

Procedure

The stock solution of Amplex Red is prepared at 0.1 M in dimethyl sulfoxide (DMSO) and stored at -20°C until use. Amplex Red is incubated at room temperature for 5 min with approximately equimolar concentrations of H_2O_2 in 50 mM Tris-HCl (pH 7.4) containing HRP (1 U/ml). The fluorescence intensity of the reaction mixture is measured with a fluorescence microplate reader equipped with a filter set for excitation and emission at 560 ± 10 and 590 ± 10 nm, respectively. This assay allows detection of as little as 5 pmol of H_2O_2 per 100-µl sample (50 nM) in a 96-well fluorescence microplate assay (Bartosz, 2006).

Note

Amplex Red is a highly sensitive and stable substrate for HRP with selectivity for H_2O_2. Resorufin is also stable and its long-wave spectrum results in reduced interference from autofluorescence in most biological samples. Given its high sensitivity, specificity, and chemical stability, Amplex Red is widely used for the measurement of low levels of H_2O_2 in various biological samples. Another advantage of this assay is that HRP is active over a wide pH range.

HRP-based spectrophotometric assay with 3,5,3′5′-tetramethylbenzidine

Background

Exposure of 3,5,3′5′-tetramethylbenzidine (TMB) to HRP and H_2O_2 results in the formation of a one-electron oxidation product, the TMB cation free radical (Pick and Keisari, 1980) (Fig. 2). The TMB cation free radical is in equilibrium with a charge-transfer complex, which is responsible for the blue color (absorbance maximum at 653 nm, with $\varepsilon = 3.9 \times 10^4$ M^{-1} cm^{-1}) that develops during TMB oxidation. Further oxidation with HRP and H_2O_2 or mild acidification of the radical yields a yellow two-electron oxidation product, the diimine (absorbance maximum of 450 nm, with $\varepsilon = 5.9 \times 10^4$ M^{-1} cm^{-1}).

Procedure for microtiter plate assay
Standards (10 μM to 1 mM H_2O_2) and samples (10 μl) are incubated for 15 min at room temperature with a reaction mixture (200 μl) containing 25 mU of HRP and 0.7 mM TMB in 0.1 M sodium phosphate buffer (pH 6.0). The reaction is terminated by the addition of 50 μl of 0.5 M sulfuric acid, and the original H_2O_2 concentration of the sample is then calculated from the absorbance at 450 nm and the standard curve.

Note
The TMB method provides a highly sensitive assay for H_2O_2. The advantages of this system include the high stability of the oxidation products of TMB at acid pH and the wide pH range over which HRP is active. TMB should be kept out of direct sunlight as it is photosensitive.

Spectrophotometric assay based on ferrithiocyanate generation

Background
Hydrogen peroxide oxidizes the ferrous ion (Fe^{2+}) to the ferric ion (Fe^{3+}), the latter of which forms a red ferrithiocyanate complex with thiocyanate (SCN^-):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$
$$Fe^{3+} + SCN^- \rightarrow FeSCN^{2+}$$

The absorbance of the ferrithiocyanate complex can be measured spectrophotometrically at 480 nm.

Procedure
For the detection of H_2O_2, 0.2 ml of 10 mM ferrous ammonium sulfate [$Fe(NH_4)_2SO_4$] and 0.1 ml of 2.5 M potassium thiocyanate (KSCN) are added to 1.0 ml of the sample containing H_2O_2. After incubation for appropriate times, the reaction is stopped by the addition of trichloroacetic acid to a final concentration of 10% (w/v). The absorbance of the purple ferrithiocyanate complex is then measured at 480 nm with a spectrophotometer.

Note
This method is simple but not very sensitive, with the extinction coefficient for $FeSCN^{2+}$ at 480 nm being only 3860 M^{-1} cm^{-1}.

Spectrophotometric assay based on the Ferrous Oxidation in the Presence of Xylenol Orange (FOX) method

Background
In dilute acid, hydroperoxides oxidize the ferrous ion (Fe^{2+}) to the ferric ion (Fe^{3+}), the latter of which reacts with xylenol orange (XO) to form a blue-purple complex, *o*-cresolsulfone-phthalein 3′,3″-bis(methylimino) diacetate, with an extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} at 560 nm, the absorbance maximum (Gay and Gebicki, 2000):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \text{ (Fenton reaction)}$$
$$Fe^{3+} + XO \rightarrow \text{blue-purple complex}$$

In addition, the hydroxyl radical generated by the Fenton reaction is scavenged by sorbitol, and the radical species so formed react with oxygen to yield a hydroperoxyl radical (Gay and Gebicki, 2000). The latter oxidizes the ferrous ion to the ferric ion, with the production of H_2O_2, which propagates the ferrous oxidation step:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$
$$\text{Sorbitol} + OH^{\bullet} \rightarrow \text{Sorbitol}^{\bullet} + H_2O$$
$$\text{Sorbitol}^{\bullet} + O_2 \rightarrow \text{SorbitolO}_2^{\bullet} \rightarrow \text{Sorbitol(keto)} + HO_2^{\bullet}$$
(peroxyl radical) (hydroperoxyl radical)
$$Fe^{2+} + HO_2^{\bullet} \rightarrow Fe^{3+} + H_2O_2$$

The presence of sorbitol increases the yield of ferric ions to ~15 mol per mole of H_2O_2. Thus, whereas the extinction coefficient of the ferric–xylenol orange complex at 560 nm is 1.5×10^4 M^{-1} cm^{-1}, the apparent extinction coefficient obtained for H_2O_2 in an optimized reaction mixture containing sorbitol is ~2.25×10^5 M^{-1} cm^{-1}. Other alcohols including methanol, ethanol, and tertiary butanol as well as DMSO also produce a color enhancement but one that is much less pronounced than that achieved with sorbitol.

Procedure
The FOX reagent contains 100 μM xylenol orange, 250 μM ferrous ammonium sulfate, 100 mM sorbitol, and 25 mM H_2SO_4; the iron salt must be dissolved directly in acid because ferrous ions are prone to auto-oxidation at neutral pH. The test sample (50 μl) is added to 950 μl of FOX reagent, mixed, and incubated at room temperature for a minimum of 30 min, at which time color development is almost complete (Jeong et al., 2000). The absorbance at 560 nm is measured after removal of any flocculated material by centrifugation. The signal is compared with an H_2O_2 standard curve and is linear in a concentration range of 0 to 5 μM. The signal is stable overnight.

Note
The FOX method is suited for the determination of low levels of H_2O_2 in aqueous media, but it can also be used to measure other water-soluble hydroperoxides such as butyl and cumyl hydroperoxides. The high apparent extinction coefficient for measurement of H_2O_2 in this assay allows the detection of 100 pmol of H_2O_2 in a 50-μl sample (2 μM). If large amounts of test sample are available, the FOX reagent can be prepared in a 20× concentrated form; in this case, 50 μl of 20× FOX1 reagent is added to 950 μl of test sample to allow measurement of hydroperoxide at concentrations as low as 100 nM. The FOX assay is relatively free from interference by other components often present in test samples (such as protein, trichloroacetic acid, and salts). There are limits, however, to the level of ascorbic acid that can be tolerated. Ferric ions cause the slow oxidation of ascorbic acid in the highly acidic FOX reagent, resulting in the generation of H_2O_2. This reaction may lead to a net oxidation of ferrous to ferric ions at high concentrations (> 50 μM) of ascorbic acid in the assay. At lower concentrations (< 20 μM), ascorbic acid may cause a net back-reduction of ferric to ferrous ions, resulting in diminution of the signal. Other components that are reducing at physiological pH (such as glutathione, other thiols, and urate) present no problem in the acidic environment of the FOX reagent. Compounds that bind ferric ions (such as desferrioxamine, diethylenetriamine pentaacetic acid, and ethylenediamine tetraacetic acid) will inevitably interfere with the reaction (through competition with xylenol orange for the generated ferric ions) and should be present at no more than 1 μM in the assay.

Detection of Intracellular H_2O_2 with 5-(and 6-)Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH-DA)

Background
2′,7′-Dichlorodihydrofluorescein (DCFH) is a colorless and nonfluorescent “dihydro” derivative of fluorescein. However, its

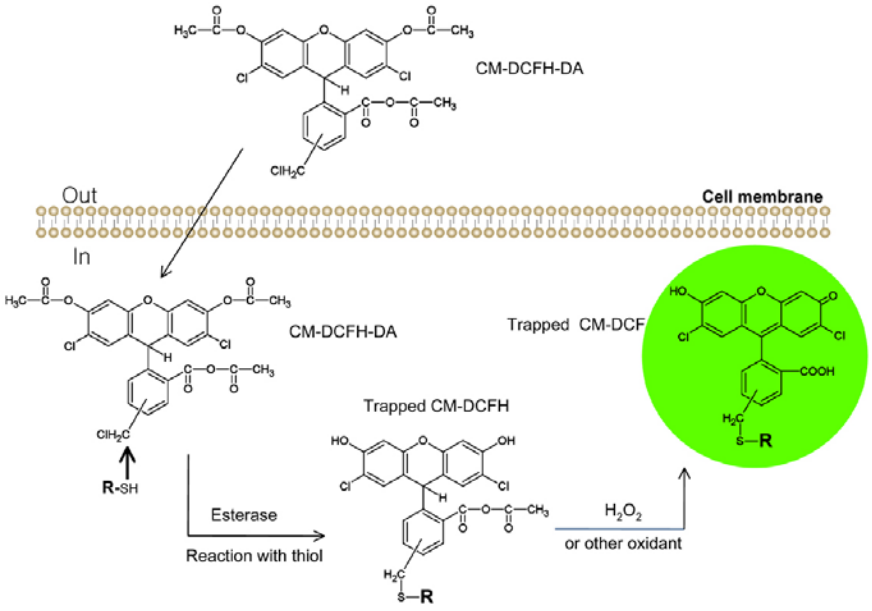

Fig. 3. Basis for the detection of intracellular H_2O_2 with CM-DCFH-DA. CM-DCFH-DA diffuses into cells and becomes trapped as a result of its deacetylation by intracellular esterases. The nonfluorescent CM-DCFH is then oxidized by various oxidants including H_2O_2 to yield the intensely fluorescent CM-DCF. In the absence of the CM (chloromethyl) group attached to the DCF moiety, oxidized DCF molecules leak out of the cell over time. However, the reactivity of the CM group with intracellular thiol components (RSH) ensures that the fluorophore is retained within the cell.

oxidation to the parent dye provides the basis for it to serve as a fluorogenic probe for the detection of ROS including H_2O_2. DCFH is not cell permeable because of its polarity, but its diacetate ester, DCFH-DA, passively diffuses into cells, where the two acetate groups are cleaved by intracellular esterases to yield DCFH. Subsequent two-electron oxidation of this trapped nonfluorescent molecule by ROS results in formation of the highly fluorescent product DCF. DCFH-DA has been used widely to measure intracellular ROS (Hempel et al., 1999). Unlike DCFH, DCF is membrane permeable and can leak out of cells. Detection of slow H_2O_2 production over time can thus be difficult with DCFH-DA (Ubezio and Civoli, 1994). To improve cellular retention of the oxidation product, a chloromethyl derivative of DCFH-DA, 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH DA), was subsequently prepared (Palomero et al., 2008). The chloromethyl group of CM-DCFH-DA allows for covalent binding to intracellular thiol components, resulting in retention of CM-DCF within the cell for longer time intervals (Fig. 3). Accumulation of CM-DCF in cells can be measured on the basis of an increase in fluorescence at ~530 nm on excitation at ~488 nm. The fluorescence can be detected with a fluorometer, flow cytometer, microplate reader, or fluorescence microscope and is thought to be proportional to the concentration of ROS in cells (Bass et al., 1983; Palomero et al., 2008; Ubezio and Civoli, 1994).

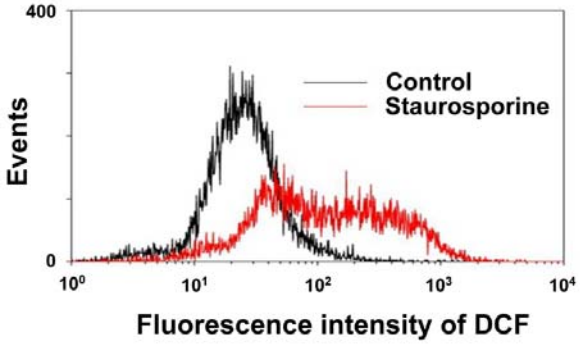

Fig. 4. Measurement of staurosporine-induced ROS production in HeLa cells by flow cytometry. HeLa cells (3×10^5) were plated in 35-mm dishes and cultured for 24 h. The cells were then incubated for 4 h in the absence (control) or presence of staurosporine (200 nM), detached from the dish, and resuspended in phenol red-free Dulbecco's modified Eagle's medium supplemented with 1% FBS. The cell suspension was loaded with 5 μM CM-DCFH-DA at 37°C for 20 min in the dark and then analyzed immediately with a flow cytometer [FACSCalibur; BD Biosciences (USA)]. A minimum of 2×10^4 cells per sample was used for each measurement with excitation and emission wavelengths of 488 and 530 nm (FL1) for CM-DCF fluorescence. Reproduced with permission from Chang et al. (2004).

General procedure

For preparation of a stock solution, CM-DCFH-DA (Molecular Probes-Invitrogen) should be reconstituted with high-quality anhydrous DMSO, dimethylformamide, or 100% ethanol. Working solutions should be freshly prepared, with any excess diluted probe discarded at the end of the work session, given that the probe undergoes oxidation in solution and the presence of moisture facilitates its decomposition. To avoid cytotoxicity, cells should be loaded with CM-DCFH-DA at low concentrations (such as 1 to 10 μM for 15 to 60 min). Higher levels of CM-DCFH-DA or high light intensities can result in artifactual photochemical oxidation to fluorescent products that can be mistakenly attributed to ROS generation. Extracellular hydrolysis of the diacetate ester can be minimized with the use of a loading buffer, such as phosphate-buffered saline (PBS), that is free of primary and secondary amines. Cells should be maintained in a medium that is free of phenol red and other colored dyes before and throughout the assay. To ensure that an added drug or other compound will not result in optical interference, its absorbance spectrum should be examined and the absorbance peak determined should not overlap with the excitation or emission peaks of the oxidized dye. Alternatively, the drug or compound can be mixed with DCF and examined for any effect on the fluorescence intensity of the dye. Basic procedures are described below for flow cytometric (Fig. 4) or fluorescence microscopic (Fig. 5) detection of CM-DCF produced in cells.

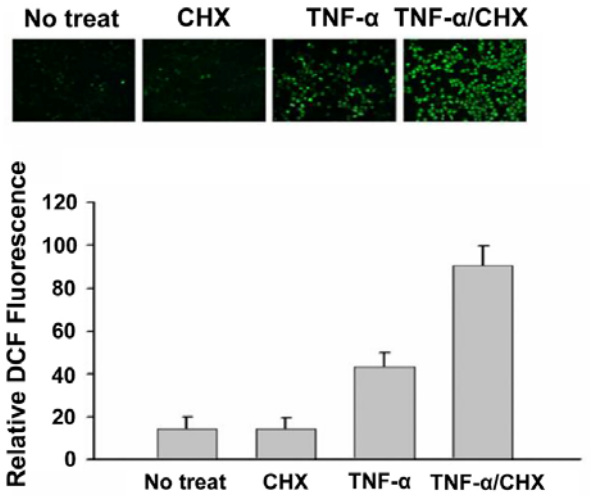

Fig. 5. Measurement of tumor necrosis factor–α (TNF-α)–induced ROS production in HeLa cells by fluorescence microscopy. HeLa cells (3×10^5) were plated in 35-mm dishes and cultured for 24 h. The cells were then deprived of serum for 6 h before incubation for 1 h in phenol red-free medium in the absence (No treat) or presence of cycloheximide (10 μg/ml, CHX), TNF-α (10 ng/ml), or both TNF-α (10 ng/ml) and cycloheximide (10 μg/ml). The cells were washed with Krebs-Ringer solution and then incubated at 37°C for 5 min with 5 μM CM-DCFH-DA in the same solution in the dark, after which CM-DCF fluorescence images (upper panels) were immediately acquired with a fluorescence microscope (Axiovert 200, Zeiss). The fluorescence intensities of 80 to 100 cells were measured and averaged with the use of a Scion imaging system (National Institutes of Health, USA). Quantitative data (lower panels) are means ± SD of fluorescence values obtained from three independent experiments. Reproduced with permission from Lee et al. (2009).

Procedure for flow cytometric detection of CM-DCF

- Shortly before (< 15 min) performing the experiment, reconstitute CM-DCFH-DA to prepare a concentrated stock solution in a vial protected from light and maintained tightly sealed until use.
- After stimulation of plated cells, isolate the cells by exposure to trypsin and wash them twice with warmed loading buffer [such as PBS, Hanks' balanced salt solution (HBSS), HEPES, or some other simple physiological buffer supplemented with 1% fetal bovine serum (FBS)].
- Resuspend the cells in warmed loading buffer and transfer them to a sterile flow-cytometer tube.
- Add CM-DCFH-DA to the cells to give a final concentration of 1 to 10 μM and then incubate the cells for 15 to 60 min at 37°C to allow loading of the probe. Keep the tube covered with foil to prevent light exposure.
- Remove the loading buffer, return the cells to a phenol red-free culture medium supplemented with 1% FBS, and incubate them at 37°C. Allow a short recovery time for cellular esterases to hydrolyze the acetate groups and render the dye responsive to oxidation. The optimal recovery time can vary widely.
- Analyze the cells with an appropriate flow cytometer at an excitation wavelength of 488 nm to measure CM-DCF fluorescence. Collect at least 10,000 events for each sample. The samples should be kept cold after loading into the machine and analyzed as soon as possible (within 30 min of dye addition).
- Perform data analysis. Plot forward versus side scatter for the cells and then a histogram of fluorescence intensity.

Procedure for direct visualization of CM-DCF by fluorescence microscopy

- Grow cells to 80% confluence on glass-bottomed 35-mm culture dishes such as those provided by MatTeK (USA).
- After stimulation, wash the cells twice with loading buffer (PBS, HBSS, HEPES, or other simple physiological buffer containing 1% FBS).
- Add CM-DCFH-DA to a final concentration of 0.1 to 5 μM and incubate the cells for 10 to 30 min at 37°C to allow loading of the probe. Keep the cells covered with foil to prevent light exposure.
- Wash the cells and resuspend them in phenol red–free culture medium supplemented with 1% FBS. Place the cells on a temperature-controlled stage of an appropriate fluorescence microscope equipped with a laser source for excitation at 488 nm. Record CM-DCF fluorescence every 15 s as soon as possible after cell staining, and then analyze the fluorescence microscopic images.

Procedure for negative controls

- For flow cytometry, ascertain that the forward and side scatter of light by cells is unchanged after dye loading and treatment. Changes in cell dimensions may be related to blebbing or shrinkage resulting from handling or a toxic response.
- Examine the fluorescence of untreated loaded cells that have been maintained in growth medium or simple buffer. In healthy cells, ROS are eliminated by cellular enzymes or natural antioxidants. After recovery from dye loading, healthy cells should exhibit a low level of fluorescence that is relatively stable for the duration of the experiment; however, a gradual increase (due to autoxidation) or decrease (due to loss of dye from cells or photobleaching) in fluorescence intensity may be observed. In the absence of any stimulus or inducement, a burst of fluorescence in healthy, untreated cells might indicate progression to cell death or some other oxidative event.

Note

The DCFH method has many advantages over other techniques developed for measurement of intracellular H_2O_2. It is easy to use, highly sensitive to changes in cellular redox state, inexpensive to perform, and suitable for monitoring changes in ROS concentration over time. However, DCFH derivatives are relatively nonselective probes that react with many oxidants such as peroxynitrite, hydroxyl radicals, lipid peroxides, nitric oxide, and hypochloride in addition to H_2O_2 (Gunasekar et al., 1995; Kooy et al., 1997). Indeed, peroxynitrite and hydroxyl radicals oxidize DCFH much faster than does H_2O_2 (Crow, 1997; Setsukinai et al., 2003). DCF fluorescence is thus a measure of generalized oxidant production rather than that of any particular reactive species; it does not provide a direct assay for H_2O_2. However, H_2O_2 is often the major ROS produced in stimulated cells, and the contribution of H_2O_2 to DCFH oxidation can be estimated from the decrease in fluorescence intensity attributable to incorporation into cells of the H_2O_2-specific enzyme catalase. Another weakness of DCFH is its instability, with DCFH derivatives being highly susceptible to photooxidation and photobleaching. Care should therefore be taken to use low-light conditions for fluorescence microscopy applications whenever possible. DCF fluorescence from intact cells is difficult to calibrate because of artifacts associated with photooxidation and photobleaching and as such can only provide qualitative information.

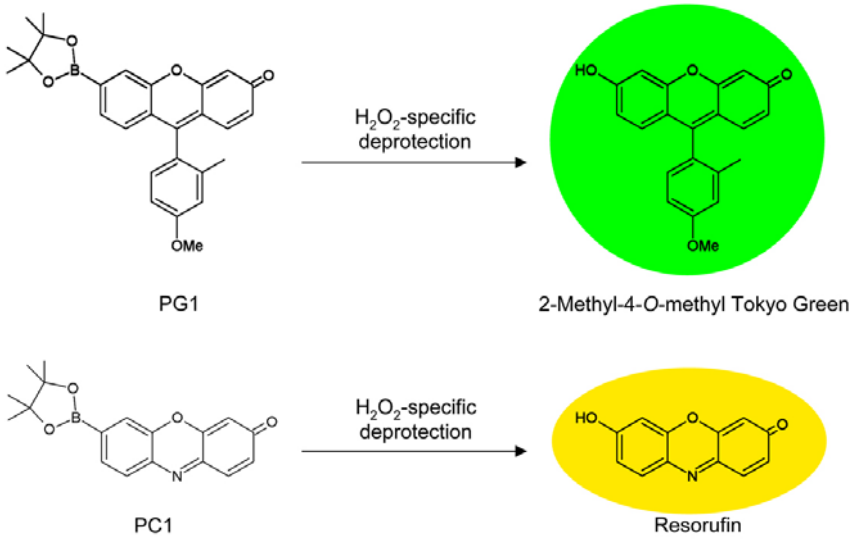

Fig. 6. Activation of the nonfluorescent probes PG1 and PC1 via an H_2O_2-dependent deprotection reaction.

Measurement of intracellular H_2O_2 with deprotection-based fluorescent probes

Background

To overcome the drawbacks of dihydro derivatives of fluorescein such as CM-DCFH-DA for the fluorometric detection of H_2O_2, which include their sensitivity to various physiological oxidants other than H_2O_2 and their susceptibility to autoxidation and photo-oxidation, research has focused on the development of an H_2O_2-specific probe whose function is not dependent on probe oxidation (Maeda et al., 2005). Two such probes, monoboronated 2-methyl-4-methoxy Tokyo Green (Peroxy Green 1, PG1) and monoboronated resorufin (Peroxy Crimson 1, PC1), have been prepared (Miller et al., 2007) (Fig. 6). PG1 and PC1 show high selectivity for H_2O_2 over other oxidants such as peroxynitrite, the hydroxyl radical, lipid peroxides, nitric oxide, and hypochloride. PG1 features one prominent absorption band in the visible region centered at 460 nm (ε = 5500 M^{-1} cm^{-1}) and shows weak fluorescence with an emission maximum at 510 nm [Φ (quantum yield) = 0.075]. PC1 has one major absorption maximum at 480 nm (ε = 4800 M^{-1} cm^{-1}), with a corresponding weak emission band centered at 584 nm (Φ = 0.006). The H_2O_2-mediated "deprotection" of PG1 and PC1 results in marked increases in green (10-fold) and red (40-fold) fluorescence, respectively, as the result of generation of 2-methyl-4-*O*-methyl Tokyo Green and resorufin, respectively (Fig. 6). PG1 and PC1 are able to enter cells, whereas the deprotection products remain restricted to the intracellular environment. PG1 and PC1 were successfully applied to the measurement of epidermal growth factor (EGF)-induced H_2O_2 generation in A431 cells and in hippocampal neurons by confocal microscopy (Miller et al., 2007).

Procedure

The procedure presented is based on that of Miller et al. (2007). A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus Glutamax-1 (Invitrogen) supplemented with 10% FBS. One day before imaging, the cells were plated on 18-mm glass coverslips. Dissociated postnatal rat hippocampal neurons (postnatal day 0 to 5) were plated at a density of 200,000 cells/ml on 12-mm, poly-L-lysine-coated glass coverslips in serum-containing medium. Probe, inhibitor, and EGF were added to cells in PBS, and the cells were incubated at 37°C for an appropriate time and then imaged. Confocal fluorescence imaging was performed with a Zeiss LSM510 NLO Axiovert 200 laser-scanning microscope and a 40× (numerical aperture, 0.8) or 63× (numerical aperture, 0.9) water-immersion objective lens. Excitation of PG1-loaded cells at 488 nm was performed with an argon laser, and emission was detected with a META detector (Zeiss) from 495 to 559 nm. Excitation of PC1-loaded cells was performed at 543 nm with a HeNe laser, and emission was detected with a META detector from 548 to 644 nm.

Note

Although PG1 and PC1 are promising tools for the selective monitoring of intracellular H_2O_2 production, they cannot provide spatial and temporal information for H_2O_2 molecules in cellular contexts. Many intracellular messengers are produced locally within the cell. In the case of H_2O_2, controlled production confined to the microdomain in which the signal must be delivered, offers clear advantages given that H_2O_2 is readily converted to toxic hydroxyl radicals inside of cells. The removal of H_2O_2 in cells is mediated predominantly by catalase, glutathione peroxidases, and peroxiredoxins, all of which are localized to various regions of the cytoplasm. Given that the catalytic efficiencies of these antioxidant enzymes range from 10^7 to 10^8 M^{-1} s^{-1} (Peskin et al., 2007), the reaction rates of PG1 and PC1 with H_2O_2 (rate constant of ~1 M^{-1} s^{-1}) are too slow for monitoring of the spatiotemporal patterns of H_2O_2 accumulation. Most of the H_2O_2 molecules produced locally would thus diffuse away from the site of production and be eliminated before reaction with the probes. Further molecular modifications that enhance the reactivity of these probes with H_2O_2 will thus be required to achieve sensitive detection at relevant thresholds. In addition, the reaction of PG1 or PC1 with H_2O_2 is irreversible. The fluorescence intensities obtained with these probes therefore reflect the cumulative total of H_2O_2 molecules produced during a given time interval; the probes cannot be used for detection of transient changes in H_2O_2 concentration.

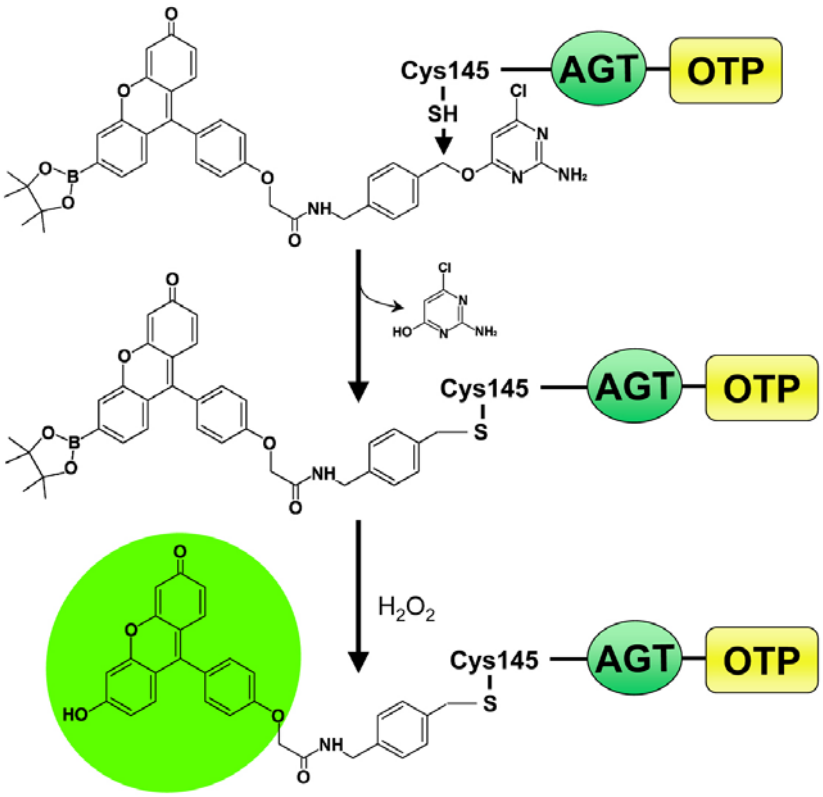

Fig. 7. Generation of organelle-specific H_2O_2 fluorescent probes by SNAP-tag conjugation chemistry. A fusion protein (SNAP-OTP) of an organelle-targeting protein (OTP) and SNAP-tag (AGT mutant) is transiently expressed in cells by transfection with an appropriate SNAP-express plasmid. Incubation of the transfected cells with the SNAP-tag substrate SPG (PG1 conjugated to benzyl-2-chloro-6-aminopyrimidine) results in its specific binding to SNAP-tag and its covalent attachment via the benzylic carbon to the thiol group of cysteine145, with elimination of the 2-chloro-6-aminopyrimidine moiety. This bioconjugation results in localization of the monoboronated fluorophore to a specific subcellular compartment determined by the OTP. Deprotection of the fluorophore by H_2O_2 results in a marked increase in green fluorescence at the target organelle.

Imaging intracellular H_2O_2 with organelle-targetable, deprotection-based fluorescent probes based on SNAP-tag protein labeling

Background

SNAP-tag® (Covalys Biosciences, Switzerland) is a 20-kDa mutant of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) that reacts specifically and rapidly with benzylguanine and benzylchloropyrimidine derivatives, thus allowing the specific covalent attachment of synthetic ligands (SNAP-tag substrates) to a fusion protein containing this tag (Gautier et al., 2008). For example, the gene for an organelle-targeting protein (OTP) can be cloned into a SNAP-express plasmid for expression of the targeting protein as a SNAP-tag fusion (SNAP-OTP). SNAP-tag has thus been fused to the carboxyl terminus of histone H2B (SNAP-H2B) for localization to the nucleus as well as to the carboxyl terminus of cytochrome c oxidase subunit 8 (SNAP-Cox8A) for mitochondrial localization (Srikun et al., 2010). The addition of the amino acid sequence KDEL, a signal sequence for protein retention in the endoplasmic reticulum, to the carboxyl terminus of SNAP-tag also resulted in localization of the fusion product (SNAP-KDEL) to this organelle. The parent SNAP-tag without a targeting sequence (pSNAP) is expressed uniformly in the cytoplasm and nucleus. A SNAP-tag substrate has been synthesized by conjugation of the deprotection-based H_2O_2 probe PG1 to benzyl-2-chloro-6-aminopyrimidine (Srikun et al., 2010). The hybrid molecule (SPG) was found to be membrane permeable. Furthermore, incubation of mammalian cells expressing a SNAP-OTP fusion protein with SPG led to the intracellular generation of an organelle-targeted fluorescent probe, SNAP-PG1, as the result of AGT bioconjugation chemistry (Fig. 7).

Procedure

The procedure presented is based on that of Srikun et al. (2010). Various SNAP-express plasmids for the expression of pSNAP, SNAP-H2B, SNAP-KDEL, or SNAP-Cox8A were obtained from Covalys Biosciences (Witterswil, Switzerland). HEK 293T cells were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine. One day before transfection, the cells were plated on four-well Lab-Tek borosilicate chamber (Nunc). Transfection with SNAP-express plasmids was performed with the use of Lipofectamine 2000 (Invitrogen). The cells were then incubated in Dulbecco's PBS containing 5 μM SPG for 30 min at 37°C, after which they were returned to DMEM with 10% FBS for 30 min to remove any unbound fluorophore. Confocal fluorescence images were subsequently obtained with the cells suspended in Dulbecco's PBS and with the use of a Zeiss LSM510 NLO Axiovert 200 laser-scanning microscope and a 40× oil-immersion objective lens. The motorized stage on the microscope was equipped with an incubator that maintained the sample at 37°C under a humidified atmosphere of 5% CO_2. Excitation of the SNAP-tag probe at 488 nm was performed with an argon laser, and emission was detected with the use of a 500- to 550-nm filter. Image analysis was performed with ImageJ software (National Institutes of Health, USA).

Note

SNAP-tag technology endows PG1 with the ability to measure H_2O_2 in living cells with subcellular resolution. Because PG1 recognition of H_2O_2 is based on deprotection rather than on oxidation chemistry, the probe is selective for H_2O_2. The deprotection reaction is irreversible, however. Various SNAP-express plasmids can be purchased from Covalys Biosciences, but the SNAP-tag substrate SPG is not yet commercially available.

Imaging of H_2O_2 with genetically encoded redox-sensitive green fluorescent proteins

Background

As discussed above, the deprotection-based probes PG1 and PC1 are selective for H_2O_2 but their reaction with H_2O_2 is irreversible, with the result that they are not suitable for detection of dynamic changes in H_2O_2 concentration. To overcome this drawback, redox-sensitive green fluorescent proteins (roGFPs), including roGFP1 (GFP with the mutations C48S, S147C, and Q204C) and roGFP2 (GFP with the same mutations as roGFP1 plus S65T), have been prepared by site-directed mutagenesis of the GFP gene (Dooley et al., 2004; Hanson et al., 2004). In an oxidizing environment, a disulfide bond is formed between Cys^{147} and Cys^{204} on adjacent β strands close to the chromophore of roGFP, and the resulting conformational change leads to an increase in the size of the excitation spectrum peak near 400 nm at the expense of that near 490 nm. The ratio of fluorescence elicited by excitation at 400 nm to that elicited at 490 nm (400/490 nm excitation ratio) is thus indicative of the extent of oxidation-that is, of the oxidant level.

Procedure for cell imaging

The procedure presented is based on that of Dooley et al. (2004). Cells were cultured in a 12-well dish containing coverslips coated with poly-L-lysine, transfected with an expression vector for roGFP, incubated for 24 to 72 h at 37°C in culture medium, and washed with HBSS. They were then imaged with a fluorescence microscope equipped with a cooled CCD (charge-coupled device) camera, and dual-excitation ratio (400/480 nm for roGFP1, 400/495 nm for roGFP2) images were collected and quantified. The fluorescence background was corrected for by manual selection of regions with unchanged fluorescence. The exposure time was 200 to 1000 ms, and images were collected every minute.

Note

Given that formation of the disulfide bond between Cys^{147} and Cys^{204} of roGFP is promoted by a variety of oxidants, the probes are not specific sensors of H_2O_2 but rather are indicators of the cellular redox state. Their genetic encoding means that the probes can be targeted to specific subcellular locations by tagging with specific targeting domains. Both roGFP1 and roGFP2 were found to be largely reduced inside cells under resting conditions (84 or 95% reduced, respectively, in HeLa cells) (Dooley et al., 2004). Exposure of cells to membrane-permeant oxidants such as H_2O_2, menadione, or diamide increased the excitation ratio within a few minutes. On washing out of the oxidant, the cells manifested a slow reduction of roGFP. The probes were able to detect the oxidant burst triggered in HL-60 cells on their differentiation into monocytes-macrophages followed by stimulation with phorbol myristic acid. However, they were not sensitive enough to detect growth factor- or cytokine-stimulated H_2O_2 production. It is also important to note that biosynthesis of the GFP fluorophore is accompanied by the generation of one molecule of H_2O_2 per molecule of mature GFP (Zhang et al., 2006). The expression of high levels of roGFP alone can thus be toxic to cells.

Imaging of H_2O_2 with the genetically encoded fluorescent indicator HyPer

Background

A H_2O_2 probe that overcomes the major disadvantage of roGFP has been prepared from a circularly permuted form of yellow fluorescent protein (cpYFP) (Belousov et al., 2006). YFP is a point mutant of GFP with a T203Y substitution that is responsible for the red-shift in emission from green to yellow fluorescence (Ormo et al., 1996). A rearranged version of YFP (cpYFP), in which the amino and carboxyl portions are interchanged and rejoined with a short spacer connecting the original termini, was shown to possess fluorescence properties that are highly sensitive to protein conformation. Fusion of calmodulin and its interacting protein M13 with the amino and carboxyl termini of cpYFP, respectively, resulted in the generation of a Ca^{2+}-sensitive fluorescent probe (Nagai et al., 2001). A highly H_2O_2-specific fluorescent probe, named HyPer, was prepared with a similar approach. OxyR is a peroxide sensor and transcriptional regulator that is able to sense the presence of H_2O_2 and induce an antioxidant system in prokaryotes (Zheng et al., 1998). OxyR contains an H_2O_2-sensing regulatory domain (OxyR-RD, amino acids 80 to 310) and a DNA-binding domain (amino acids 1 to 79). Formation of an intramolecular disulfide bond between residues Cys^{199} and Cys^{208} in the regulatory domain results in a conformational change that activates the OxyR transcription factor. HyPer was prepared by inserting cpYFP into OxyR-RD with short amino acid linkers (Fig. 8A). The fluorescence spectrum of HyPer has two excitation maxima at 420 and 500 nm and an emission maximum at 516 nm (Fig. 8B). On exposure to H_2O_2, the excitation peak at 420 nm decreases while that at 500 nm increases, thus qualifying HyPer as a ratiometric sensor. These changes in fluorescence properties are due to oxidation of OxyR-RD as evidenced by the observation that they were prevented by replacement of Cys^{199} or Cys^{208} in the OxyR-RD portion of HyPer with Ser. HyPer appears to be selective for H_2O_2, with neither nitric oxide, peroxynitrite, the superoxide anion, nor oxidized glutathione being able to induce changes in HyPer fluorescence.

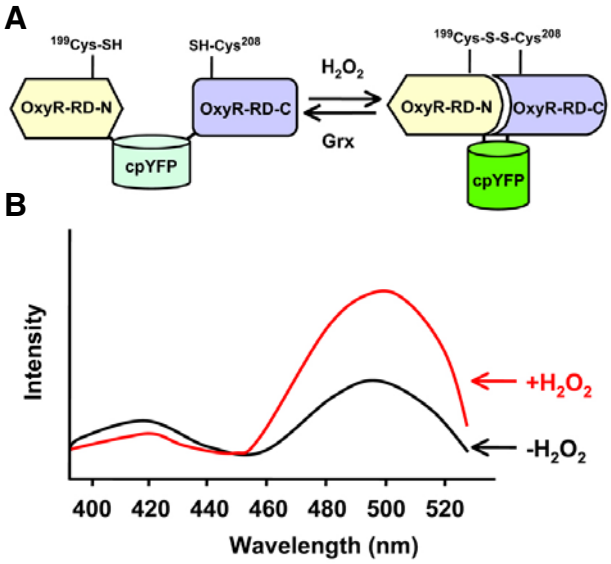

Fig. 8. Detection of H_2O_2 with HyPer. (A) Domain structure of HyPer-C (cytosolic HyPer). The conformational change attributable to the H_2O_2-induced formation of a disulfide bond between Cys^{199} and Cys^{208} located in the amino (N) and carboxyl (C) portions of the regulatory domain of OxyR (OxyR-RD), respectively, drives a ratiometric fluorescence change of cpYFP. The reverse reaction is catalyzed by glutaredoxin (Grx). (B) Excitation spectra of HyPer at neutral pH before and after oxidation by H_2O_2. Emission was measured at 530 nm.

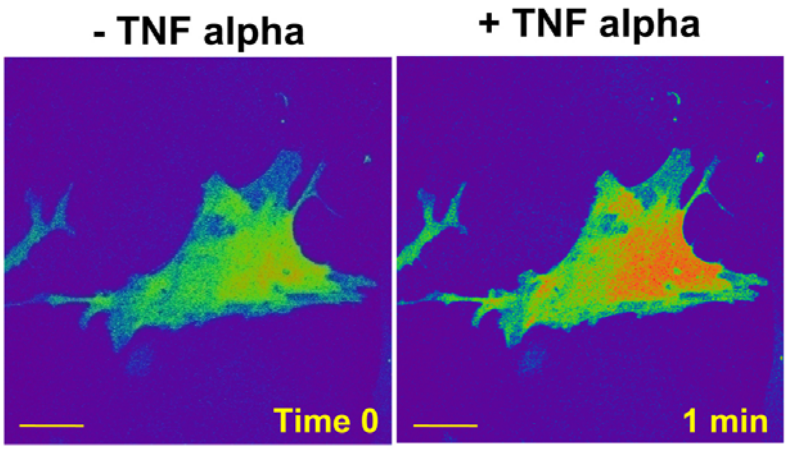

Fig. 9. Detection of TNF-α–induced H_2O_2 production in rat aortic smooth muscle cells with HyPer. Cells expressing cytosolic Hyper were stimulated with TNF-α (50 ng/ml) for 1 min, and fluorescence was imaged with a confocal microscope and with excitation and emission at 488 and 500 to 530 nm, respectively. The images were obtained from seven focal planes in the *z*-axis and merged at 0 and 1 min. Scale bar, 20 μm.

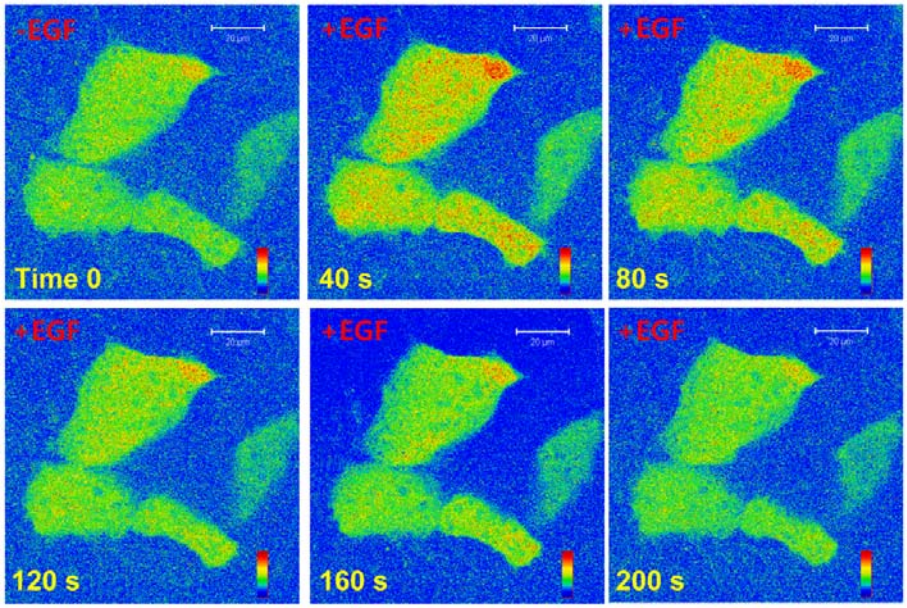

Fig. 10. Dynamics of EGF-induced H_2O_2 production in A431 cells measured with HyPer. A431 cells expressing cytosolic HyPer were stimulated with EGF (200 ng/ml) for the indicated times, after which images were obtained with a confocal microscope and with excitation and emission at 488 and 500 to 530 nm, respectively. The images were obtained from five focal planes in the *z*-axis and merged at each time point. Scale bar, 20 μm.

Procedure for cell imaging

The presented procedure is based on that of Belousov et al. (2006) and Markvicheva et al. (2008). Cells were cultured in 12-well dishes containing coverslips (diameter, 12 mm) coated with poly-L-lysine and were transfected with an expression vector for Hyper with an appropriate transfection reagent. After incubation for 18 to 24 h at 37°C in culture medium, the cells were washed with HBSS before imaging and analysed with a Nikon A1R confocal microscope and NIS elements AR 3.0. Fluorescence was excited with a 488-nm laser and detected at 500 to 530 nm. Alternatively, the cells can be observed with an Olympus US SZX12 fluorescence stereomicroscope or Olympus CK40 fluorescence microscope with an Olympus DP50 camera. The dual-excitation ratio images were acquired and quantified with the use of D405/40X and SZX-FGFP BP469-490 excitation filters (Chroma Technology). Emission can be detected at 500 to 530 nm. Images were collected at one frame per 40 s or every minute. For calculation of Hyper ratio images, images obtained with excitation at 488 or 405 nm were corrected for background and divided. Examples of confocal microscopic measurement of intracellular H_2O_2 with HyPer are shown in Figs. 9 and 10.

Note

HyPer is a reversible sensor of H_2O_2 and, like roGFP, can be targeted to a specific subcellular compartment. Unlike roGFP, however, HyPer reacts with H_2O_2 selectively and is sensitive enough for ratiometric fluorescence imaging of H_2O_2 in HeLa cells stimulated with EGF or in PC-12 cells stimulated with nerve growth factor (Belousov et al., 2006). Mitochondrial H_2O_2 generation during apoptosis induced by Apo2L (TRAIL) in HeLa cells was also detected with a mitochondrially targeted form of Hyper (Belousov et al., 2006; Markvicheva et al., 2008). A HyPer probe was introduced into zebrafish embryos by injection of mRNA to monitor H_2O_2 dynamics in response to local injury of the tail fin (Niethammer et al., 2009). The mammalian expression vectors pHyPer_Cyto (for HyPer expression in the cytosol), pHyPer_dMito (for mitochondrially targeted HyPer), and pHyPer_nuc (for nuclear targeted HyPer) can be purchased from Evrogen (Russia).

CONCLUSIONS

Over the last 5 years or so, methods for the detection and measurement of intracellular H_2O_2 have improved substantially with regard to the availability of chemical or genetically encoded probes. In particular, much needed selectivity for H_2O_2 over other cellular oxidants has been provided by switching the H_2O_2-sensing mechanism from oxidation- to deprotection-based mechanisms for chemical probes and by the development of OxyR-based genetically encoded probes. However, the selectivity of chemical probes has been gained at the expense of sensitivity, given that the deprotection reaction is relatively slow, with the result that most of the H_2O_2 produced in cells is destroyed by cellular antioxidant systems before it can react with the probe. Further molecular modifications that enhance the reactivity of such probes with H_2O_2 will be required to achieve sensitive detection at relevant thresholds. The ultimate goal is to obtain reversible probes that are able to monitor dynamic changes in H_2O_2 concentration. As of now, no reversible chemical probes are available for H_2O_2. The genetically encoded probe HyPer is a reversible sensor that qualifies for ratiometric measurement and can be targeted to various subcellular compartments. The OxyR-based sensing mechanism of HyPer appears to be fast enough to compete with cellular antioxidant systems. Given that it is derived from GFP, however, HyPer is less fluorogenic than are chemical probes derived from DCF or resorufin. Also, being a derivative of GFP, the biosynthesis of HyPer is accompanied by generation of one molecule of H_2O_2 per molecule of mature fluorophore. The expression of HyPer at high levels can itself therefore alter the intracellular level of H_2O_2.

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