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Enhanced detection of H_2O_2 in cells expressing Horseradish Peroxidase

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Abstract
A new procedure for fluorescent detection of intracellular H_2O_2 in cells transiently expressing the catalyst Horseradish Peroxidase (HRP) is setup and validated. More specific reaction with HRP largely amplifies oxidation of the redox probes used (2′,7′-dichlorodihydrofluorescein and dihydrorhodamine). Expression of HRP does not affect cell viability. The procedure reveals MAO activity, a primary intracellular H_2O_2 source, in monolayers of intact transfected cells. The probes oxidation rate responds specifically to the MAO activation/inhibition. Their oxidation by MAO-derived H_2O_2 is sensitive to intracellular H_2O_2 competitors: it decreases when H_2O_2 is removed by pyruvate and it increases when the GSH-dependent removal systems are impaired. Specific response was also measured after addition of extracellular H_2O_2. Oxidation of the fluorescent probes following reaction of H_2O_2 with endogenous HRP overcomes most criticisms in their use for intracellular H_2O_2 detection. The method can be applied for direct determination in plate reader and is proposed to detect H_2O_2 generation in physio-pathological cell models.

Keywords: *Horseradish Peroxidase, intracellular H_2O_2, ROS sensitive probes, MAO activity, transfection, cell signalling*

Abbreviations: *Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein diacetate; DHR, dihydrorhodamine 123; HRP, Horseradish Peroxidase; ROS, reactive oxygen species; MAO, monoamine oxidases; BSA, bovine serum albumin; FCS, foetal calf serum; tyr, Tyramine; clorg, Clorgyline; depr, Deprenyl; HRP^+, HRP positive cells; WT, wild type; DTPA, di-ethylentriamine pentacetic acid*

Introduction

Reactive oxygen species (ROS), so far mainly known for the role played in pathological conditions, including cancer, diabetes mellitus, ischemia, neurodegeneration, and ageing [1,2], are also seen today as driving signals for physiological events. Among ROS, the most, and possibly the sole, physiologically relevant species in signalling is hydrogen peroxide. This non-radical species, indeed, specifically interacts with targets, such as 'labile' thiols, producing a functionally relevant redox switch [3,4].

From compelling experimental evidence, H_2O_2 emerges as a messenger in signalling pathways, eventually leading to proliferation, metabolic adaptation and apoptosis [4]. Formed by several aerobic dehydrogenases or following the dismutation of superoxide, H_2O_2 can rise from the steady-state nanomolar range [5].

The best known mechanism for the reduction of dioxygen to superoxide, precursor of hydrogen peroxide, is the activation of NADPH oxidases, although also mitochondria could be relevant in this respect [6,7].

The foremost relevant information for studying the pathological, as well as the signalling and regulatory functions of H_2O_2, is its actual concentration in living cells, for which simple reliable methods are still lacking. To this purpose, ROS sensitive fluorescent probes, increasing fluorescence emission following a crucial redox transition have been largely used [8]. These molecules do not directly react with hydrogen peroxide, but require

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the catalysis of a heme-peroxidase [9]. The peroxidase provides the specificity for both the oxidant and the probe. The most used probes are the membrane permeable diesters of 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) and Dihydrorhodamine 123 (DHR).

Although widely adopted, the use of redox sensitive probes for detecting H_2O_2 in intact cells has been criticized in several respects [8–10]: (a) other hemoproteins such as cytochrome c, free heme and iron itself can catalyse the oxidation of the probes, also independently of the presence of H_2O_2; (b) cellular reductants such as GSH can compete with the probes for the oxidizing species; and (c) the probes are prone to undergo an auto-oxidation, primed by photo-irradiation, during fluorescent microscopy observation, yielding superoxide and thus producing an artifactual increase of H_2O_2.

Eventually, these measurements, requiring a microscope or a very sensitive detection system, are not suitable for processing a large number of samples. Moreover, the results are resistant to the robust validation necessary to quantitatively compare different experimental conditions.

To increase the sensitivity of the detection of H_2O_2 in cells and to minimize the actual relevance of the pitfalls arising from competing reactions, we set up an innovative procedure where the catalyst of the oxidative reaction is Horseradish Peroxidase (HRP) transiently expressed in mammalian cells.

This could overcome a large part of the above drawbacks related to specificity, while allowing, at the same time, direct measurements in plate readers escaping from cell selection and side-effects of the photo-irradiation microscopy.

To induce an increase of intracellular H_2O_2 and to test the analytical procedure, activation of the constitutive Monoamine Oxidases was the strategy used. These flavoproteins (MAOs; EC 1.4.3.4) belong to the class of aerobic dehydrogenases catalysing the oxidation of a substrate and the reduction of molecular oxygen to hydrogen peroxide. Monoamine oxidases are largely expressed in mammalian tissues [11,12] and are located in the outer mitochondrial membrane. Since the flow of H_2O_2 produced by MAO can be easily manipulated in cells by substrate availability and specific inhibitors, its measurement is suitable for testing and validating the analytical procedure proposed in this study. Two isoforms of MAOs are known (A and B), differing in substrate and inhibitor specificity [13–15]. MAO A preferentially deaminates serotonin, adrenaline and noradrenaline and is irreversibly inhibited by clorgyline [15,16]. MAO B preferentially deaminates β-phenylethylamine and is inhibited by deprenyl [16,17]. Tyramine and dopamine are substrates for both isoforms.

To date, the MAO-dependent H_2O_2 production has been directly measured only in MAO-enriched sub-cellular preparations or in tissue homogenates [18–20]. The only report in intact cells deals with concentrated cell suspensions where H_2O_2 release was detected as luminol-HRP amplified chemiluminescence [21,22].

We intend to verify the HRP expression procedure as a reliable way to detect H_2O_2 in intact cells directly following the oxidation of the common intracellular fluorescent probes DHR and $DCFH_2$, overcoming some limits and criticisms in their use.

Materials and methods

Reagents and stock solutions

Fatty acid free Bovine Serum Albumin (BSA), DMEM phenol red free, Horseradish Peroxidase (E.C. 1.11.1.7), glucose oxidase from *Aspergillus Niger* (E.C. 1.1.3.4) resazurin, monobromobimane, Propidium Iodide, MTT (3-(4,5- dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromid), CDNB (1-chlor-2 ,4-dinitrobenzene) and BSO (buthionine sulphoximine or S-(n-buty1)homocysteine sulphoximine) were from Sigma (St. Louis, MO). The fluorescent probes Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine), DHR (dihydro-rhodamine 123), $DCFH_2$-DA (2′,7′-dichlorodihydro fluorescein diacetate) and Hoechst 33342, DAPI (4,6-diamidino-2-phenylindole) were purchased by Molecular Probe, Inc. (Eugene, OR), dissolved in analytically pure DMSO and stored at -20°C. The BCA protein assay (23227) was from Pierce (Rockford, IL) and the Cytotoxicity detection kit (LDH) (11 644 793 001) was from Roche (Roche Diagnostic S.P.A., Milano, Italy). Lipofectamine^{TM} 2000, trypsine, DMEM (41966-029), OptiMEM and FCS were obtained from Invitrogen (Carlsbad, CA). Goat anti-HRP Tetramethyl Rhodamine conjugated (TRITC) antibody was from Jackson ImmunoResearch laboratories (Pennsylvania).

The incubation medium was KRB (118,5 mM NaCl, 4,74 mM KCl, 2,5 mM $CaCl_2$, 1,18 mM KH_2PO_4, 1,18 mM $MgSO_4$) supplemented with 20 mM HEPES, 5 mg/ml BSA and 25 mM glucose pH 7.4. All other chemicals from commercial sources were of analytical grade.

Cell culture and incubation conditions

C_2C_{12} (ATCC Nr: CRL-11268) and SHSY-5Y (ECACC Nr. 85051005) adherent cells were grown in an humidified thermostat chamber at 37°C and 5% CO_2 in complete DMEM (medium with 4500 mg/l glucose supplemented with 10% (v/v) FCS) unless otherwise specified. Cultures were grown to 70–80% confluence. Experiments either in transfected or WT cells were usually performed, unless otherwise stated, in the KRB incubation medium.

Lipofection procedure

The efficiency of transfection performed was optimized adopting an inverted procedure. C_2C_{12} and SHSY-5Y were transfected with Lipofectamine™ 2000 and ssHRP-KDEL pRK5 plasmid in 25 cm^2 polystyrene flasks. The HRP containing plasmid ssHRP-KDEL pRK5 [23] was a generous gift of professor D. F. Cutler. The DNA (125 μg/ml) and the Lipofectamine (250–350 μg/ml) were each prediluted in OptiMEM. The 1:1 (v/v) DNA/Lipofectamine mixture prepared was used to pre-treat polystyrene flasks (DNA was omitted in vehicle-treated cells) before seeding cells suspended in complete DMEM medium after trypsinization. The medium was removed after 18 h and replaced with fresh complete DMEM. At 24 h cells were trypsinized and seeded at the desired concentrations on polistyrene multiwell plates or on coverslips for the HRP immunodetection. Transfection efficiency was calculated as percentage of immunodetected HRP positive cells/number of nuclei stained with 10 μM Hoechst dye or with DAPI. At least five fields/sample at low resolution were considered.

HRP protein immuno-detection

Cells were fixed in cold methanol:acetone: H_2O (2:2:1) for 10 min at $-20^{\circ}C$ and samples presaturated in PBS containing 1% BSA were incubated with anti-HRP TRITC conjugated antibody 1:100 in saturating buffer for 1 h. After three washes in PBS and one in H_2O, cover slips were treated with a drop of ProLong® Gold antifade reagent containing nuclear stain, mounted on a slide and observed under a fluorescent microscope.

Propodium iodide staining

After 4 h at $37^{\circ}C$ cells were treated for 20 min more with 20 μM Propidium Iodide and 10 μM Hoechst. The permeability to the PI nuclear stain, measured by fluorescent microscopy imaging, was used as index of cell death and reported as mean ± SD percentage of PI stained nuclei/total (Hoechst stained) nuclei [24]. Data were calculated from the corresponding images acquired by a Zeiss fluorescent inverted microscope implemented with the Image J software. At least three fields/sample in triplicate conditions at 80 × magnification were considered.

Lactate dehydrogenase released

The measurement of lactate dehydrogenase released from cells [25] was performed with the Cytotoxicity Detection Kit (Roche). LDH released (% total) is expressed as mean ± SD of triplicate analysis.

Metabolic activity

Cell viability was assessed with MTT assay [26] based on the reduction of the methyl tetrazolium salt to the purple formazan by mitochondrial activity. The MTT test was performed after 30 min of incubation in complete DMEM with 1 mg/ml of MTT followed by the colourimetric determination of the formazan salt formed as net absorbance (540–620 nm) of DMSO solubilized samples and reported as a percentage of the corresponding WT cells.

Protein assay

The micro-method of BCA protein assay was used on aliquots of Triton X100 cell lysates essentially as described by the producer and measured at 540 nm at the plate reader (Multiscan XE Thermo Labsystems).

Cell GSH depletion

The GSH content was lowered by enzymatic coniugation with CDNB (1-chlor-2,4-dinitrobenzene) [27] or by inhibiting *de novo* biosynthesis with buthionine sulphoximine [28]. In both cases preincubations (1 h with 30 μM CDNB and 14–16 h with 1 mM BSO) were performed in complete DMEM and excess reagents removed before the experiments.

Radiochemical assay for MAO A activity

The oxidase activity, was measured using [^{14}C] 5-hydroxytryptamine as substrate in intact C_2C_{12} cells, using the procedure previously described [21]. Briefly, near confluent C_2C_{12} cells in six wells plates (10^6 cells/well) were incubated in an humidified thermostat chamber at $37^{\circ}C$ in the KRB containing BSA buffer for 12 h with 5-hydroxytryptamine (0.3 mM, 0. 5 μCi/mM); with or without the MAO A inhibitor clorgyline (0.3 μM) present also in 30-min of preincubation. Supernatants and 0.1% SDS cell lysates were separately collected and the reaction stopped with 0.5 N HCl. Reaction products were extracted by addition of three volumes of solvent (toluene/ethyl acetate, v/v). The radioactivity present in the organic phase was counted in a β-scintillation counter. The sum (supernatant + lysates) of recovered labelled serotonin metabolites found in viable C_2C_{12} cells (~10 nmoles of serotonin were oxidized/hour by 10^6 cells) accounted for MAO A activity being 95% inhibited in the presence of clorgyline.

H_2O_2 production by amine oxidase activity in permeabilized cells

Hydrogen peroxide production from Amine Oxidase activity was measured with Amplex Red/HRP [29] in a plate reader [30]. H_2O_2 production was followed as increase of the fluorescent (at 544/590 nm) resorufin formed by the oxidation of the non-fluorescent

Amplex Red (40 μM) in the presence of HRP (50 mU/ml). Cells were treated with tyramine (1 mM), clorgyline (0.3 μM) and deprenyl (1 μM). When present, clorgyline and deprenyl were pre-incubated for at least 30 min before tyramine additions. The Amplex Red oxidation rate was recorded before and after permeabilization with 0.1% Triton X100. At the end of experiments titrated amounts of H_2O_2 were added as internal standard for calibration.

Activity of the expressed HRP protein

The activity of the expressed HRP protein was measured in total cell lysates with 0.1% Triton X100, recording the oxidation of 40 μM Amplex Red following addition of 20 μM H_2O_2 at 544/590 nm. The rates of fluorescence increase before and after additions of H_2O_2 were measured and reported as mean pmoles H_2O_2 titrated/min/8000 cells.

DHR or $DCFH_2$ detection of the MAO-dependent hydrogen peroxide production in intact cells

Ninety-six wells plates with HRP transfected or WT cells (8×10^3/well) were used on the fluorescent plate reader or under inverted fluorescent microscopy at $80\times$ magnification. At zero time the complete DMEM was replaced with the incubation medium containing the fluorescent probe (10 μM $DCFH_2$-DA or 10 μM DHR). Tyramine (1 mM) was added to start H_2O_2 production. When present, the MAO inhibitors were added 30 min before tyramine and fluorescent probes.

Direct readings (10 min cycles) were performed in triplicate on a Fluoroskan Ascent FL plate reader at 485–527 nm, the oxidation rate was calculated and data reported as arbitrary units of fluorescence increase (AUF/min) mean of triplicate analyses in 100 min.

Fluorescence microscopy was performed after 3 h of incubation. One shot (at fixed exposition time of 2–3 s) images were acquired at the Zeiss inverted fluorescence microscope in the different experimental conditions soon after washing away reactants and replacing medium with fresh incubation medium. Images acquired with the Metamorph software were analysed by the Image J software for the fluorescence signals. Reported data represent the total cell intensity as means $\pm$ SD of at least 200–300 cells/field for every condition.

Statistical analysis

All data are presented as mean $\pm$ SD in at least triplicate samples. Where appropriate, Student's *t*-tests were performed, to give significance values.

Results

H_2O_2 produced in cells by monoamine oxidase does not reach the extracellular space

In agreement with a previous report [31], we confirmed in SHSY-5Y and found in C_2C_{12} cells a prevalent MAO A activity. The MAO A activity was also confirmed in intact C_2C_{12} cells measuring labelled metabolites product of [^{14}C] 5–hydroxytryptamine oxidative deanimation.

HRP and the most sensitive probe Amplex Red added to the culture medium fail to detect any hydrogen peroxide in cells exposed to MAO substrates unless they are permeabilized (Figure 1A), clearly indicating that H_2O_2 produced within the cells by MAO activity does not reach the extracellular compartment. While confirming the production of H_2O_2 by MAO, in both cell lines (in Figure 1B the rates obtained in both cell lysates are summarized), this evidence points out the efficiency of intracellular H_2O_2 scavenging systems. The larger inhibitory effect of clorgyline over deprenyl on H_2O_2 production suggests that in both cell lines MAO A prevails over MAO B.

The detected background oxidation of the probe in the absence of MAO substrates and insensitive to inhibitors is reasonably accounted for by other intracellular sources of H_2O_2.

Efficiency of HRP transfection and protein expression

Cells were transfected with the HRP coding plasmid using the inverse lipocationic procedure described under Methods. The efficiency of the exogenous expression procedure was tested both by immunofluorescent detection of the protein and measurements of the activity. The transfection efficiency obtained by the transfection protocol we set up (50% in SHSY-5Y and 30% in C_2C_{12}) was higher than that obtained by the classic lipocationic procedure (less than 10% in SHSY-5Y [32] and 3% in C_2C_{12} [33]).

The percentage of HRP positive cells is maximal at 29–48 h, when it starts to decrease following cellular duplication (Figure 2A). Anyway, the actual number of HRP positive cells/field remains constant for at least the next 2 days.

The HRP activity increases up to 72 h (Figure 2B). Remarkably, the activity of HRP at 48 h strictly correlates with the number of immunodetected transfected cells and this permits the extrapolation to 100% transfected cells. A value of ~ 90 nmoles H_2O_2 reduced/min was the HRP activity normalized to 1×10^6 transfected cells in both SHSY-5Y and C_2C_1 as measured by the Amplex Red oxidation rate with standard H_2O_2 addition. Calculation are based on the 1:1 Amplex Red:H_2O_2 stoichiometry in the

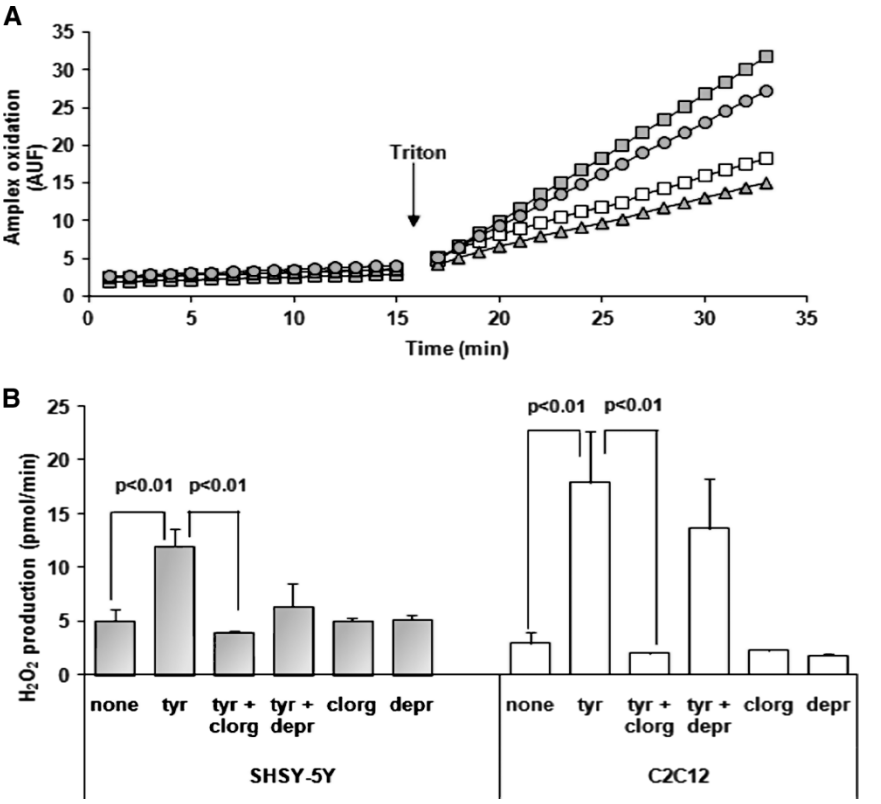

Figure 1. Hydrogen peroxide production by MAO activity. (A) The fluorescence increase of Amplex Red oxidation in the presence of HRP was followed at the plate reader. At 15 min 0.1% Triton X100 was added to C_2C_{12} cells. The fluorescence increase was measured in untreated cells (□); or in the presence of the MAO substrate Tyramine (1 mM) (■); to which were added the MAO inhibitors, 300 nM clorgyline (▲) or 1 □M deprenyl (●). In (B) are summarized the corresponding H_2O_2 production rates as pmoles/min, calculated from the oxidation rate of Amplex Red with known H_2O_2 standard by $\sim 8 \times 10^3$cells, in both C_2C_{12} and SHSY-5Y cells. Relevant statistical differences are indicated.

presence of HRP [29]. This value testifies similar expression of HRP in SHSY-5Y and C_2C_{12} and can be used to normalize data obtained in different transfected preparations.

Cell viability and metabolism in HRP transfected cell

The percentage of propidium iodide (PI) permeable cells (Figure 3A, empty bars) and the LDH release (Figure 3A, gray bars) are not affected by HRP transfection. The same holds for the redox metabolic activity of cells, measured as MTT reduction (Figure 3B). For both the death index and redox metabolic activity, the minimal and not statistically relevant negative effect induced by Lipofectamine is not worsened by HRP expression.

Intracellular MAO activity can be monitored as oxidation of an intracellular redox sensitive probe in HRP transfected cell

In HRP transfected SHSY-5Y and C_2C_{12} cells observed by fluorescence microscopy, the oxidation of the intracellular DHR increased following the addition of the MAO substrate tyramine (Figure 4), while no increase was detectable in WT cells. The inhibition of the DHR oxidation in the presence of clorgyline supports the specificity for MAO A of the observed production of the oxidant H_2O_2. Similar results have been obtained using $DCFH_2$-DA as intracellular probe (not shown).

Since fluorescent microscopy analysis is not suitable for multiple analyses and carries on practical disadvantages such as long time of acquisition and likely photoreaction artefacts, we adopted the strategy of performing a direct fluorimetric detection in a multiwell plate reader. This can be obtained from any single transfection experiment where the highly transfected preparation in batch, after trypsinization at 28 h, is homogeneously seeded typically in a 96 wells plates with less than 10% variability in the amount of transfected cells/well.

The fluorescent probes DHR and $DCFH_2$ were added to transfected cells, in parallel with the corresponding WT, and their oxidation recorded in a fluorescence plate reader, following addition of MAO substrates and inhibitors.

A faster oxidation rate of both DHR (Figure 5A) and $DCFH_2$ (Figure 5B) was detected upon tyramine addition in HRP transfected C_2C_{12} cells. The lower not relevant rates obtained in the corresponding WT

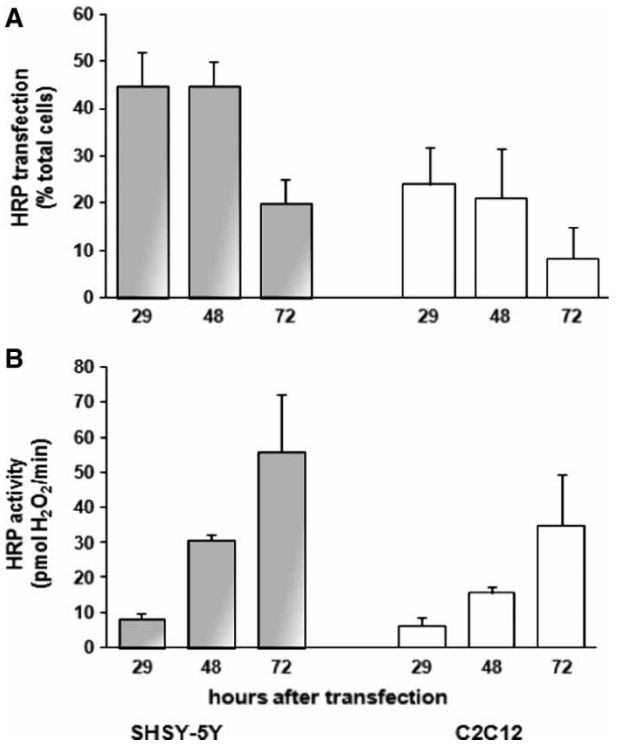

Figure 2. Efficiency of transfection (A) and expression of HRP (B) in C_2C_{12} and SHSY-5Y cells. (A) The percentage of immunostained HRP positive cells is reported at different times following transfection. (B) Activity of the expressed HRP, calculated as in Figure 1 in the presence of Triton X100, is reported as pmoles H_2O_2 titrated/min/8×10^3 cells.

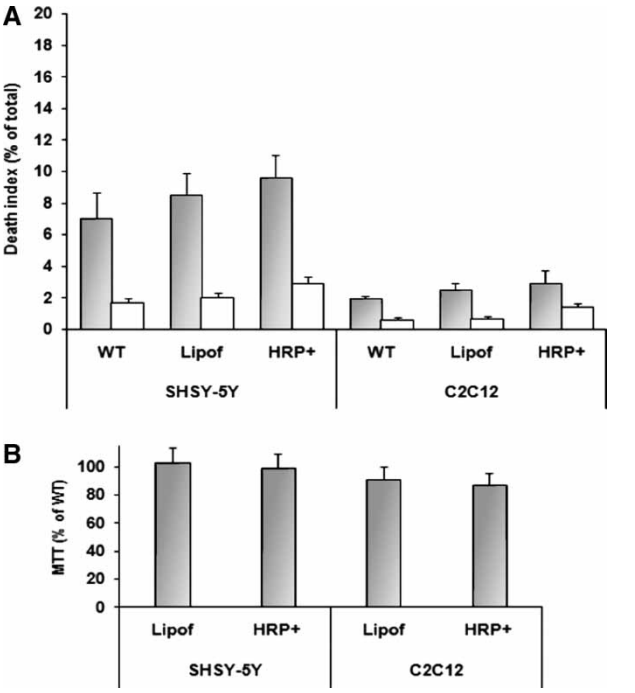

Figure 3. Transfection with HRP does not affect cell viability. (A) The cell death index as percentage of LDH release (grey bars) and PI staining (white bars) in cells transfected with HRP or treated with Lipofectamine alone is reported with the corresponding WT. In (B) the MTT reduction (index of redox metabolic activity) is reported for both cell lines as a percentage of the corresponding value of WT. Data in triplicate are representative of three independent preparations.

directly indicate the amplification of the signal obtained by transfection. As expected, clorgyline decreased the probes oxidation rate, while deprenyl showed minor inhibition. The discrepancy with the results obtained on cell lysates (Figure 1) where deprenyl was not inhibitory could depend on some cross-inhibition of deprenyl in intact cells also on MAO A, as already reported [34]. Similar data were obtained in SHSY-5Y (not shown).

The use of cells transfected with HRP for measuring intracellular H_2O_2 was further validated by titrating the diffusion to the cytoplasm of H_2O_2 generated in the culture medium. As shown in Figure 6A and B, with DHR and $DCFH_2$, respectively, in HRP transfected SHSY-5Y cells the addition of glucose oxidase to the glucose containing medium produced a dose-dependent increase of the rate of oxidation of the intracellular probe, reaching a maximum at 200 nmoles/min of H_2O_2 production (as independently measured by Amplex Red/HRP test). In these experiments the metal ions chelator DTPA (1 mM) was present in the external medium to minimize the artifactual oxidation of DHR or $DCFH_2$ (the same results were obtained in C_2C_{12}, not shown).

The specificity for H_2O_2 of the oxidation of intracellular probes was confirmed using pyruvate

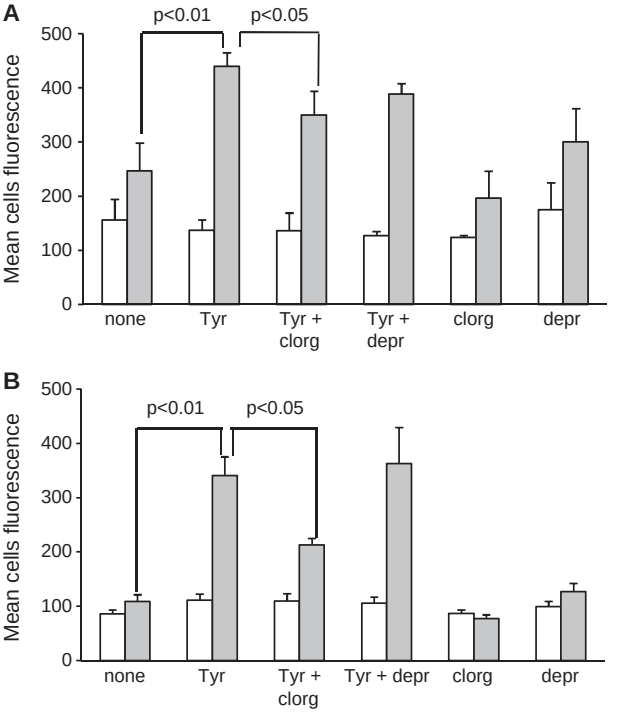

Figure 4. Detection by fluorescence microscopy of DHR oxidation in SHSY-5Y (A) and C_2C_{12} cells (B). Rhodamine mean fluorescence in cells was measured as described under methods on transfected (grey bars) and WT cells (white bars) in the presence, when indicated, of MAO substrate and inhibitors as in Figure 1. Relevant statistically different data are indicated.

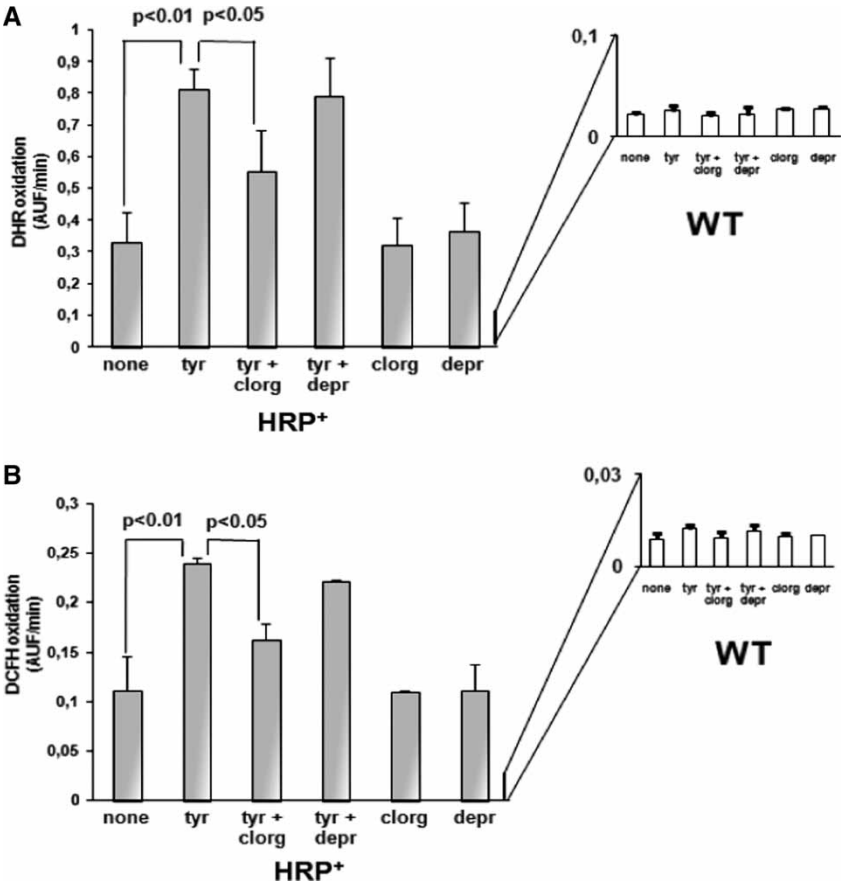

Figure 5. Direct determination of the MAO-induced DHR (A) and $DCFH_2$ (B) oxidation rate using a fluorescent plate reader. MAO-dependent H_2O_2 production in HRP transfected and WT C_2C_{12} is reported as oxidation rate of DHR or $DCFH_2$. Cells were treated with tyramine, clorgyline and deprenyl as in Figures 1 and 4. Relevant statistically different data are indicated of at least three different preparations in triplicate.

as a competitor of HRP [35], as shown in Figure 7A and B. Pyruvate, indeed, easily enters in cells, probably by means of the monocarboxylate transporters, where it interacts with H_2O_2 [36]. The nucleophilic attack of the mono-deprotonated peroxide species (HO_2^-) at the C-2 carbonyl group carbon centre of pyruvate brings decarboxylation to acetate [36]. This reaction occurs stoichiometrically with H_2O_2 at physiological pH and pyruvate is the most efficient scavenger of H_2O_2 among several

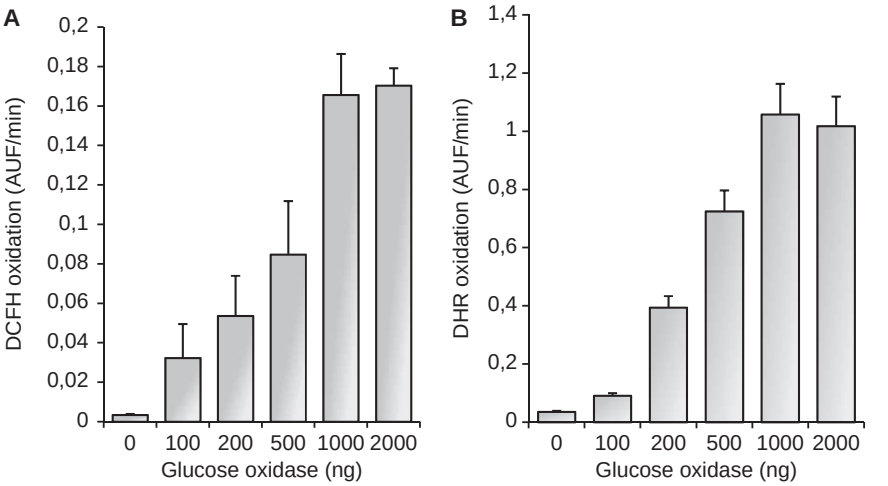

Figure 6. Oxidation rate of redox probes in HRP transfected cells depends on exogenous H_2O_2. The oxidation rate of $DCFH_2$ (A) and DHR (B) in SHSY-5Y cells was recorded using a plate reader in the presence of the indicated amount of glucose oxidase to generate extracellular H_2O_2.

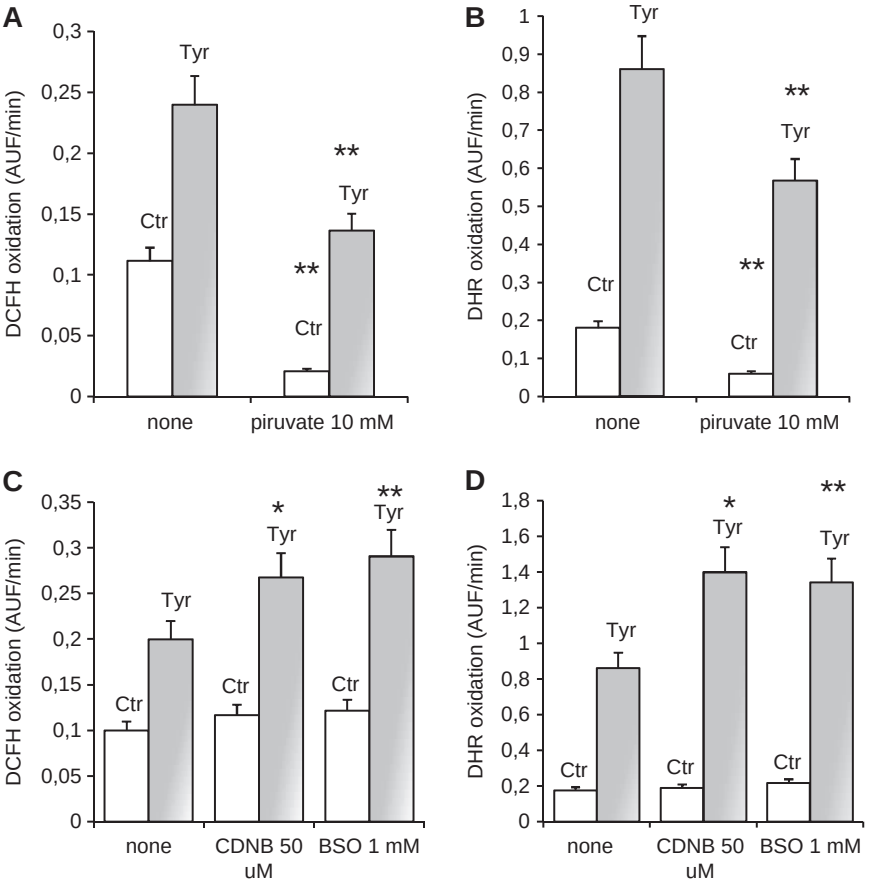

Figure 7. Positive evidence that the oxidation rate of redox probes in HRP transfected cells depends on H_2O_2. The oxidation rates of $DCFH_2$ (A, C) and DHR (B, D) with or without tyramine in SHSY-5Ycells was measured in plate reader. In (A, B) addition of the H_2O_2 scavenger pyruvate is reported. In (B, D) cells pre-treated with CDNB or BSO, to lower GSH concentration, are compared with the corresponding untreated cells. $^{\star}p<0.05$, $^{\star\star}p<0.01$ of treated (pyruvate, CDNB or BSO) vs the respective conditions in untreated cells.

physiological α cheto-acids [35–39]. As expected, in the presence of 10 mM pyruvate, the rates of $DCFH_2$ (Figure 7A) and DHR oxidation (Figure 7B) at basal level and following tyramine addition are deeply lowered.

An opposite effect is expected for agents decreasing the efficiency of endogenous H_2O_2 removal systems. To test this, we used 1-chlor-2,4-dinitrobenzene (CDNB), both acting as GSH depleting agent as substrate of glutathione-S-transferase [27] and as inhibitor of the thioredoxin reductase at even lower concentrations [40]. We also used buthionine sulphoximine (BSO) as an inhibitor of GSH biosynthesis [28]. Both these compounds (Figure 7C and D, $DCFH_2$ and DHR, respectively), by limiting the efficiency of the removal of hydroperoxides, increased the rate of oxidation of intracellular probes. This indicates that in transfected cells HRP competes with cellular peroxidases for the reduction of MAO produced H_2O_2 and provides further specificity for the assay.

Unfortunately, the relevance in this respect of catalase could not be tested since the aminotriazole, the most efficient inhibitor of catalase, inhibits also HRP.

The detection of intracellular H_2O_2 production requires a minimal number of transfected cells

To find out which is the minimal amount of transfected cells required to run reliable H_2O_2 measurements experiments we prepared mixed cultures diluting transfected cells with WT. The percentage of HRP positive cells for each mixture was tested in parallel by immuno-detection. In both cell lines the oxidation rate of both probes was a linear function of the percentage of transfected cells. In Figure 8 are reported data relative to SHSY-5Y: with DHR (Figure 8A) 10% of HRP positive cells suffice to show a statistically significant increase (Figure 9A, B) of the oxidation rate upon tyramine addition while with the less efficient fluorescent $DCFH_2$ (Figure 8B) a 20% is needed. Similar results were obtained in C_2C_{12} (not shown) where the minimum level required with DHR was 5%. These results indicate a limit of sensitivity of the procedure of $\sim$400–1000 transfected cells/well to reveal the MAO activity.

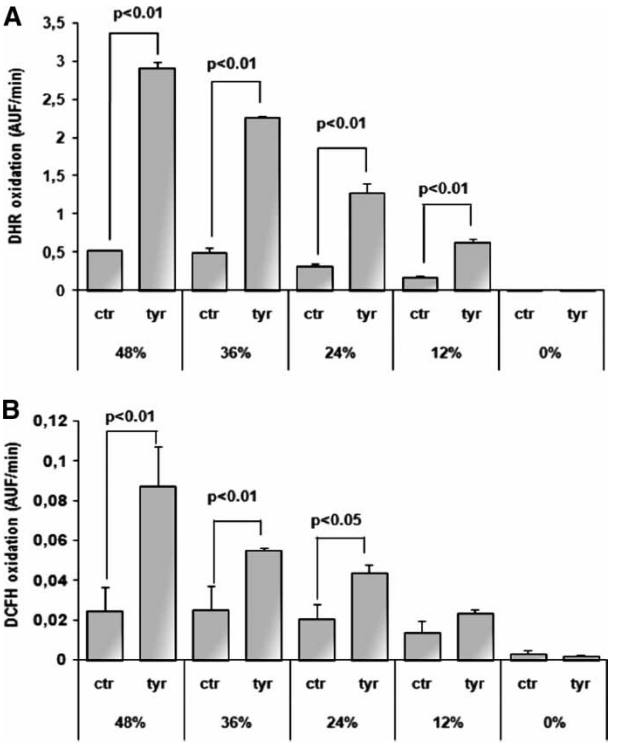

Figure 8. Detection of intracellular H_2O_2 formation requires a minimal percentage of transfected cells. Oxidation rates of DHR (A) and $DCFH_2$ (B) in HRP^+ SHSY-5Y cells mixed with WT cells are reported at the indicated HRP^+ percentage. Tyramine was 1 mM. Data in triplicate are representative of three independent preparations. Relevant statistically different data are indicated.

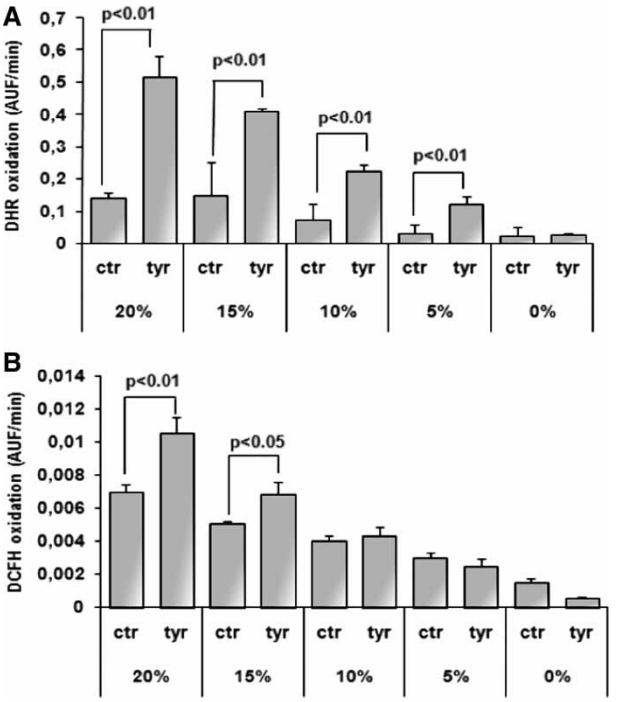

Figure 9. Detection of intracellular H_2O_2 formation requires a minimal percentage of transfected cells. Same as in Figure 8, but in C_2C_{12} cells.

Other minor H_2O_2 sources could require higher transfection efficiency. Eventually, from total HRP activity and/or percentage of transfection, the rate of H_2O_2 production can be normalized for comparative studies in different preparations.

Discussion

Hydrogen peroxide is the relatively stable product of two electron reduction of molecular oxygen. This 'reactive oxygen species' is generated in the cells by specific flavo-enzymes (aerobic dehydrogenases) or from dismutation of the superoxide differently produced by NAD(P)H oxidases and mitochondria. Besides the intracellular sources, hydrogen peroxide produced in the extracellular space can also diffuse back to the cytosol, where it participates in the unbalance between oxidants and scavenging systems known as oxidative stress.

After having been seen just as a damaging event, the formation of H_2O_2, emerges nowadays as a player of the redox regulation of signalling pathways.

In this scenario the careful, accurate and direct measurement of the cellular concentration of H_2O_2 is highly desirable.

The most suitable approach is based on the detection and recording of the oxidation of intracellular probes such as $DCFH_2$ and DHR. The redox transition, however, is not direct, but requires a catalyst that is first oxidized by H_2O_2, forming a transient species able in turn to oxidize the probe which changes its spectroscopic features. The limiting factors for the application of this analytical approach are the concentration in the cells of a haemoprotein competent for the catalysis of the oxidation and the long time of irradiation required for the acquisition under the microscope.

The present work has been inspired by the criticisms to the use of redox sensitive intracellular probes as sensors of H_2O_2 in intact cells pointing out a limited sensitivity and specificity [10].

We addressed the issue of increasing the sensitivity and the specificity of the detection of intracellular H_2O_2 by introducing the use of cells transfected with HRP. Since a much larger part of H_2O_2 is metabolized by HRP, sensitivity and specificity both increase. Also the pitfall of unknown and possibly variable stoichiometry between DHR and $DCFH_2$ oxidation and H_2O_2 [10], if not fully overcome, is definitely less relevant. In the presence of HRP, indeed, the specific reaction rate largely increases and prevails over the background of unspecific oxidative chain reactions.

The high sensitivity achieved, eventually, permits the substitution of a fluorescence microscope with the much more convenient fluorescence micro plate readers, suitable for multiple analyses.

Using the Lipofectamine procedure, as modified in our protocol, the transfection efficiency is particularly

high and the excellent correlation between the number of transfected cells and signal recorded permits a calibration extrapolated to 100% of transfection efficiency. No apparent toxicity of the transfection procedure has been observed, thus the procedure can be referred to as safe.

The procedure was tested by measuring H_2O_2 diffusing to the cytosol from the extracellular compartment and was applied to the direct determination of MAO activity in living cells. Notably, this activity has never been directly measured before in intact cells and was not detectable in non-HRP transfected cells.

The specificity for H_2O_2 was confirmed by the effect of pyruvate that chemically scavenges H_2O_2 and thus depresses the oxidation of the probes. Consistently, also the increased oxidation rate of the probe following GSH depletion supports the specificity of the detection system. The probe is oxidized at a faster rate when the GSH dependent removal of H_2O_2 in prevented, although the possibility that GSH also competes with the probe for the oxidized form of HRP cannot be positively ruled out.

The inhibition of the basal level of oxidation in the presence of pyruvate indicates that the measurements could be ideally applied to any intracellular H_2O_2 source.

Using a simple fluorescent plate reader, kinetic measurements (by repeated short reading cycles) of hydrogen peroxide can be performed in as low as 400–1000 HRP transfected cells. This is crucial for long lasting experiments, when limiting the stress of cells maintained in a thermostat chambers is appropriate.

The procedure we propose is suitable for studies where H_2O_2 is investigated as a factor in cell damage or as a signalling molecule, when experimental conditions require a long time for conditioning or pre-treatments and a large number of samples has to be processed.

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