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Research Paper

Insights into the HyPer biosensor as molecular tool for monitoring cellular antioxidant capacity

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ABSTRACT

Aerobic metabolism brings inexorably the production of reactive oxygen species (ROS), which are counterbalanced by intrinsic antioxidant defenses avoiding deleterious intracellular effects. Redox balance is the resultant of metabolic functioning under environmental inputs (i.e. diet, pollution) and the activity of intrinsic antioxidant machinery. Monitoring of intracellular hydrogen peroxide has been successfully achieved by redox biosensor advent; however, to track the intrinsic disulfide bond reduction capacity represents a fundamental piece to understand better how redox homeostasis is maintained in living cells.

In the present work, we compared the informative value of steady-state measurements and the kinetics of HyPer, a H_2O_2-sensitive fluorescent biosensor, targeted at the cytosol, mitochondrion and endoplasmic reticulum. From this set of data, biosensor signal recovery from an oxidized state raised as a suitable parameter to discriminate reducing capacity of a close environment. Biosensor recovery was pH-independent, condition demonstrated by experiments on pH-clamped cells, and sensitive to pharmacological perturbations of enzymatic disulfide reduction. Also, ten human cell lines were characterized according their H_2O_2-pulse responses, including their capacity to reduce disulfide bonds evaluated in terms of their migratory capacity.

Finally, cellular migration experiments were conducted to study whether migratory efficiency was associated with the disulfide reduction activity. The migration efficiency of each cell type correlates with the rate of signal recovery measured from the oxidized biosensor. In addition, HyPer-expressing cells treated with N-acetyl-cysteine had accelerated recovery rates and major migratory capacities, both reversible effects upon treatment removal. Our data demonstrate that the HyPer signal recovery offers a novel methodological tool to track the cellular impact of redox active biomolecules.

1. Introduction

Antioxidant consumption is a widely acquired nutritional behavior. Underlying the justification of this conduct is the promise that high antioxidant intake prevents oxidative damage, thus avoiding a variety of diseases associated with an intracellular redox imbalance [1,2].

The antioxidant property of some molecules is currently assessed by evaluating their capacity to protect a target molecule from oxidation in an abiotic environment [3]. Several constraints arise to the use of this widespread analytical process. First, the antioxidant property is evaluated without considering cells (i.e., far from interactions with cellular components that affect the reactivity of the tested molecule). Second, the chemical-based procedure to analyze antioxidant properties consists of merely exposing the target molecule to a unique interaction with the antioxidant; a nonexistent situation in the intracellular environment, where antioxidants must deal with a variety of cellular components that can exert a collaborative effect with the tested molecule. And third, by their nature, antioxidant chemical analyses are limited to a reduced time of action, even though the antioxidant inside the cellular environment is exposed to many sequential reactions over a longer time, and with the chance of affecting long-term cellular defense mechanisms.

The combination of molecular biology and imaging techniques has created many genetically-encoded fluorescent biosensors to follow intracellular parameters in living cells, overcoming the majority of the limitations related with redox chemical analyses depicted above [4].

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Typically, a biosensor is a fusion protein composed by a motif that recognizes a specific molecule, usually taken from bacteria, and assembled with one or two fluorescent proteins. Upon binding of the target molecule, a conformational change is induced that modifies the spectral properties of the fluorescent domains. Some sensors have two fluorescent domains with overlapping emission and excitation spectra, capable of undergoing Förster resonance energy transfer (FRET) and responding to the molecule with a change in FRET efficiency [5].

A biosensor for tracking intracellular hydrogen peroxide in living cells was developed in 2006 by Lukianov et al. [6]. The probe, HyPer, consists of a circularly permuted yellow fluorescent protein (cpYFP) inserted into the regulatory domain of the *Escherichia coli* H_2O_2-sensing protein OxyR. The properties of wild type OxyR, such as specificity and sensitivity to H_2O_2, are conserved in this tool, which has been useful to detect H_2O_2 variations under growth factor stimulation in mammalian cells [7] and to resolve spatial and temporal oxidation events in Tau-HyPer HeLa cells [8]. As bacterial OxyR, HyPer biosensor does not respond to oxidized molecules derived from nitrosative stress. Although S-Nitrosothiols are able to react with Cys-199 at OxyR [9], the reaction does not proceed to form a disulfide bond [10], a requirement to achieve conformational change in the biosensor, which limits the capacity of HyPer to monitor nitrosative stress events. Despite of this limitation, this molecular tool allows overcoming one of the main disadvantages of dichlorofluorescein (DCF) based methods to measure cellular reactive oxygen species (ROS), since DCF produces ROS under light exposure [11].

The use of HyPer has been concentrated in measuring levels of H_2O_2 in living cells under receptor activation and fisiopathological conditions both in mammalian [12–14] and plant cells [15–17]. Intracellular H_2O_2 levels are driven primarily from enzymatic reductions of superoxide anions, carried out by superoxide dismutases (SODs). Mitochondrial metabolism is one of the main contributors of superoxide anion production, specifically as a marginal electronic leakage through the electron chain transport [18]. Less contributing processes, which deal with electron transference, are xanthine oxidases and NADPH oxidase, among others. All these observations have helped to conceive a heterogeneous spatio-temporal distribution of intracellular H_2O_2 according to the subcellular site of generation, the nature of triggering stimulus and the abundance of antioxidant systems dedicated to neutralize peroxide excess [18–21].

On the other hand, much less attention has been put in the cellular processes involved in the recovery of oxidized HyPer. Disulfide bond reduction formed on proteins is probably carried out by Thioredoxin Reductase/thioredoxin or Glutathione Reductase systems. In this line, it has been proposed that Hyper reduction could be a useful method to assess the antioxidant activity in living cells [19,20]. In this study, we have analyzed HyPer signal during and after the application of pulses of hydrogen peroxide in order to dissect biosensor responses. Recovery signal rates from an oxidized state discriminated better the reducing properties of three subcellular compartments (cytosol, mitochondrion and endoplasmic reticulum) than steady-state measurements. Our study includes pH dependency of HyPer parameters as a baseline, the response to H_2O_2 and the recovery from an oxidized state on pH-clamped cells, a technique that conserves cellular integrity and ensures intracellular pH control.

Our results allowed us to gain information about the antioxidant capacity of the cytoplasmic environment in terms of the efficiency of counterattacking an incoming oxidant bolus and the velocity to restore disulfide bonds formed on the biosensor. We also connected the biosensor signal recovery rate with the migration potential by determining that cell lines that presented the highest recovery HyPer rates also had the highest migratory capacity. Moreover, we observed that by increasing the reductive tone of cells by adding an exogenous cysteine donor, like N-acetyl-cysteine, also resulted in a major migratory capacity. Our results indicate that HyPer recording can become a useful tool for reporting changes in antioxidant capacity in the environment and is suitable for evaluating the cellular impact of compounds with antioxidant potential.

2. Materials and methods

Reagents and salts for buffer preparation were purchased from Sigma-Aldrich (MO, USA). Lipofectamine® 2000, media culture and supplements were acquired from Life Technologies (NY, USA). EUK-134 was purchased from Cayman Chemical Company (MI, USA). PX-12, nigericin, and valinomycin were acquired from Tocris (MO, USA). BCECF-AM was acquired from Thermo Fisher Scientific (MA, USA). The 30% hydrogen peroxide solution used in this work was acquired from Merck (Darmstadt, Germany).

2.1. Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC): HTB-45™, epithelial cells from human kidney adenocarcinoma (A704); HB-8065™, epithelial cells from human hepatocellular carcinoma (HepG2); CRL-8621™, astroglia derived from human fetus brain corresponds to SV40 transformed cells (SVGp12); HTB-81™, brain metastatic epithelial cells derived from human prostate carcinoma (DU145); CRL-2086™, human fibroblasts derived from breast carcinoma (CCD-1068SK) and HTB-37™, epithelial cells from human colorectal adenocarcinoma (Caco-2) were cultured in MEM-supplemented 10% fetal bovine serum. CCL-18™, epithelial cells from lung carcinoma (A549) required F-12K Medium, supplemented with fetal bovine serum to a final concentration of 10%. HTB-22™, epithelial cells from pleural effusion derived from human mammary gland adenocarcinoma (MCF-7) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum. Ad-293 cells, derived from HEK-293 cells with improved adherence properties, were kindly donated by Dr. Diego Varela (Universidad de Chile) and were cultured in Eagle's Minimum Essential Medium, supplemented with 0.01 mg/ml human recombinant insulin and 10% fetal bovine serum. CRL-2310™, keratinocytes from human fetus correspond to papillomavirus transformed cells (CCD-1102 KERTr) were grown in Keratinocyte-Serum-Free Medium, with added keratinocyte supplements, including bovine pituitary extract (Gibco, TermoFisher Scientific, USA) and human recombinant epidermal growth factor (Gibco, TermoFisher Scientific, USA), further supplemented with an additional 30 ng/ml human recombinant epidermal growth factor (BD Biosciences, USA). CRL-4025™, endothelial cells from neonatal dermic microvasculature (TIME) were cultured in Vascular Cell Basal Medium, supplemented with the Microvascular Endothelial Cell Growth Kit-VEGF and 12.5 µg/ml blasticidine. In general terms, cells were maintained under a humidified atmosphere with 5% CO_2/air, and culture media was renewed every 2 or 3 days. When 70 – 80% of confluence was reached, cultures were expanded to other plaques or seeded on glass coverslips to be further imaged. At this point, it is necessary to state that all cell types were used within passage numbers 3–15 where no significant changes in baseline values were found (data not shown).

2.2. Adenoviral particles production and infection

Adenoviral vectors were generated using the AdEasy system [21]. Briefly, cyto-HyPer cDNA (Evrogen, Moscow, Russia) was sub-cloned into the commercial adenoviral vector pAdEasy-RFP using conventional molecular biology techniques, while homologous recombination was performed using BJ5183 cell transformation. Recombinant adenoviral plasmids were transfected in Ad-293 cells with lipofectamine, following manufacturer guidelines. Following observation of cytopathic effects (CPE), usually after 14–21 days, cells were harvested and subjected to three freeze-thaw cycles, followed by centrifugation to remove cellular debris. The resulting supernatant (2 ml) was used to infect a 10 cm dish of 90% confluent Ad-293 cells. Following CPE observations after