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Switching on a transient endogenous ROS production in mammalian cells and tissues

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ABSTRACT

There is a growing interest in the physiological roles of reactive oxygen species (ROS) as essential components of molecular mechanisms regulating key cellular processes, including proliferation, differentiation and apoptosis. This interest has fostered the development of new molecular tools to localize and quantify ROS production in cultured cells and in whole living organisms. An equally important but often neglected aspect in the study of ROS biology is the development of accurate procedures to introduce a ROS source in the biological system under study. At present, this experimental requirement is solved in most cases by an external and systemic administration of ROS, usually hydrogen peroxide.

We have previously shown that a photodynamic treatment based on the endogenous photosensitizer protoporphyrin IX and further irradiation of the target with adequate light source can be used to transiently switch on an *in situ* ROS production in human cultured keratinocytes and in mouse skin *in vivo*. Using this approach we reported that qualitatively low levels of ROS can activate cell proliferation in cultured cells and promote a transient and reversible hyperproliferative response in the skin, particularly, in the hair follicle stem cell niche, promoting physiological responses like acceleration of hair growth and supporting the notion that a local and transient ROS production can regulate stem cell function and tissue homeostasis in a whole organism. Our principal aim here is to provide a detailed description of this experimental methodology as a useful tool to investigate physiological roles for ROS *in vivo* in different experimental systems.

1. Introduction

Reactive oxygen species (ROS) are oxygen containing molecules characterized by a high chemical reactivity. In mammals, ROS are typically produced during the aerobic metabolism as a result of the reduction of molecular oxygen (O_2) by molecular hydrogen (H_2) from reduced biomolecules giving water (H_2O) [1]. The coupling of this redox reaction with the production of ATP (oxidative phosphorylation) driven by the mitochondrial electron transport chain (ETC) is the main source of biochemical energy in aerobic organisms [2,3]. However, oxidative phosphorylation is a leaky process, and 0.2–5% of the electrons circulating through the ETC in each round of ATP production incompletely reduce O_2 to give superoxide radicals ($\cdot O_2^-$) [4]. Thus, normal mitochondrial ETC functioning generates a steady production of $\cdot O_2^-$, constituting a major site of incidental ROS production inside the cell. In mammalian cells, $\cdot O_2^-$ molecules are also actively produced in a dedicated way by NADPH oxidases (NOX) and to a lesser extent as by-products of a number of metabolic reactions involving cyclooxygenase1/2, lipoxygenase, xanthine oxidase and cytochrome p450 enzymatic activities [5–7].

Superoxide radicals are anions at physiological pH so that their diffusion through biological lipidic membranes is rather limited [8]. As a consequence, the superoxide anion is sequentially reduced inside the cell to give hydrogen peroxide and hydroxyl radical. Peroxyl and alkoxyl radicals as well as hypochlorite ions are also produced in the course of the superoxide anion reduction in biological systems [6,9,10]. All these ROS types can result

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extremely harmful for cell viability through the oxidation and subsequent functional inactivation of several cell components, including polyunsaturated fatty acids (lipid peroxidation), amino acids and different enzyme co-factors [6,9,10]. ROS can also irreversibly modify the DNA molecule, severely disturbing DNA replication and transcription mechanisms [9,10]. All these processes can activate irreversible apoptotic and necrotic cell death mechanisms.

At a systemic level, the oxidative stress induced by an abnormal intracellular production and/or accumulation of ROS has been associated with several human diseases. Most of these disease states, including neurodegenerative (Parkinson's, Alzheimer's and Huntington's diseases), psychiatric (schizophrenia and bipolar disorder) and cardiovascular (stroke and myocardial infarction) disorders [10,11], the sickle cell disease and the fragile X and chronic fatigue syndromes [11,12], severely impairs human welfare. In parallel with its implication in pathological states, the oxidative stress or free radical theory of aging proposes a causal link between gradual, time-dependent increase in ROS production/accumulation during the whole lifetime and aging of the organism, a controversial theory that remains the subject of intense debate [13,14].

2. ROS signalling

In this scenario, eukaryotic organisms have developed a coordinated battery of biochemical mechanisms to efficiently manage the harmful effects of ROS, maintaining a strict balance in the cell between ROS production and the antioxidant pool. Members of the superoxide dismutase (SOD) enzymatic family catalyze the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide. Different catalases and peroxidases further catalyze the conversion of hydrogen peroxide to water and molecular oxygen using different components of the antioxidant pool (glutathione, NADPH, peroxiredoxin, thioredoxin) to maintain the equilibrium between reduced/oxidized species [15]. However, despite the actual toxic nature of ROS, reflected in the trend of most biological systems to rapidly balance excessive ROS production rates, these molecules can act as true second messengers. There are several reports indicating that eukaryotic cells can also actively promote the production of small amounts of ROS as part of signalling pathways that regulate cell survival and proliferation and as a mechanism of defense against pathogens [5,8,15,16]. In this sense, eukaryotes have developed enzymatic systems, well-illustrated by the NOX family, which are specifically dedicated to the production of superoxide radical with physiological signalling purposes [7].

Most of the experimental work to characterize the molecular mechanisms regulating ROS biology in eukaryotic cells has been done by using established cell culture lines as model systems. In a step forward, different *in vitro* 3D and animal models have been recently used in an effort to establish the physiological roles of ROS *in vivo*. Thus, it has been shown that local changes in ROS concentration are associated to the differentiation program of hematopoietic progenitors in *Drosophila* [17]. In zebrafish, a ROS gradient in the tissue is formed upon wound induction [18] and, in a similar way, a transient ROS production occurs during wound tail regeneration in *Xenopus* tadpoles [19]. Recent reports have also pointed out roles for ROS in the regulation of stem cell activity in mammals. The exogenous administration of hydrogen peroxide can have a stimulatory effect on the self-renewal and differentiation of neural stem cells in a neurosphere model [20]. It has also been shown that the deregulation of intestinal stem cell proliferation that occurs during colorectal cancer initiation is consistently associated with a differential ROS production [21]. In mouse skin, impairment of mitochondrial activity by a conditional deletion of the mitochondrial transcription factor A (TFAM) results in defective hair follicle growth [4], indirectly suggesting a role for ROS in the modulation of hair follicle stem cells. All these observations imply the existence of widespread ROS-dependent mechanisms for the regulation of cell function and tissue homeostasis. In this sense, the emergence of ROS as potential second messengers with important signalling properties has fostered the development of molecular tools to detect and quantify ROS production in cells, tissues and whole organisms.

3. ROS detection and measurement

Giving the high reactivity and chemical instability of ROS, detection and measurement of these molecules under physiological conditions poses different experimental hindrances. Adequate ROS sensors are essential tools to analyze the physiological roles of these unstable molecules. Optimal sensors should have chemical specificity for ROS molecules, a rapid reversible kinetics, a high signal-to-noise ratio and a good rate of intracellular loading [22,23]. At present, available biological ROS sensors are fluorescent beacons that fall into two categories, small-molecule or protein-based probes.

The most widely used ROS sensors are the synthetic small molecules 2′-7′-dichlorodihydrofluorescein (2H-DCF) and dihydroethidium (DHE) [22,23]. The diacetate derivative of 2H-DCF (2H-DCF-DA) is a highly cell membrane permeable compound that, upon cellular uptake, is rapidly converted to 2H-DCF by the action of intracellular esterases. The non-fluorescent 2H-DCF form reacts with different ROS giving the fluorescent compound 2′-7′-dichloro fluorescein (DCF) that can be further detected by fluorescence microscopy, FACS, spectrofluorometry and related experimental approaches. DHE is also a cell permeable molecule that preferentially reacts with $\cdot O_2^-$ giving the highly red fluorescent product 2-hydroxyethidium that is rapidly accumulated in the cytoplasm [24]. Inside living cells, DHE can also react with other ROS, mainly hydrogen peroxide, giving ethidium cation molecules, a red fluorescent DHE-derived species that specifically intercalates between the DNA bases thereby increasing its fluorescent emission. There are other small molecules that can be efficiently used as ROS sensors, including the boronate-based multicolor peroxy sensor family, dihydrorhodamine 123, HKGreen-3 and other rhodol-based fluorescent probes [22,25,26]. As a rule these small molecules can be effectively used to track and quantify ROS production under stringent and defined experimental conditions. However, important caveats should be taken into consideration when designing experiments that imply the use of this type of compounds. For instance, many of these molecules are chemically promiscuous and can react with different ROS and other intracellular highly reactive species containing nitrogen, are light sensitive or can further produce ROS in unspecific feedback reactions [22].

In the last years, different genetically engineered fluorescent protein probes have been used to analyze specific parameters related to ROS biology, including redox-oxidation sensitive green fluorescent proteins (roGFPs) for redox potential measurement [27], the HyPer construct family to quantify H_2O_2 production [28,29] and the mt-circularly permutated yellow fluorescent protein (cpYFP) to quantify mitochondrial $\cdot O_2^-$ flashes [30]. As compared to small molecule indicators, these protein probes have improved selectivity with respect to ROS types. These molecules can also be specifically targeted to specific cell types or tissues, by using adequate promoters, or to different cellular compartments by fusing the sensor cassette with target-specific molecular domains. Furthermore, this technology has been used to design and produce transgenic animals allowing a partial *in vivo* visualization of ROS dynamics [19,29,31]. However, although promising, these fluorescent protein probes have different limitations that