Journal of Molecular and Cellular Cardiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Review article

Imaging dynamic redox processes with genetically encoded probes

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ARTICLE INFO

Article history:
Received 15 November 2013
Received in revised form 23 December 2013
Accepted 27 December 2013
Available online xxxx

Keywords:
Genetically encoded redox probes
Hydrogen peroxide
Superoxide
roGFP
cpYFP

ABSTRACT

Redox signalling plays an important role in many aspects of physiology, including that of the cardiovascular system. Perturbed redox regulation has been associated with numerous pathological conditions; nevertheless, the causal relationships between redox changes and pathology often remain unclear. Redox signalling involves the production of specific redox species at specific times in specific locations. However, until recently, the study of these processes has been impeded by a lack of appropriate tools and methodologies that afford the necessary redox species specificity and spatiotemporal resolution. Recently developed genetically encoded fluorescent redox probes now allow dynamic real-time measurements, of defined redox species, with subcellular compartment resolution, in intact living cells. Here we discuss the available genetically encoded redox probes in terms of their sensitivity and specificity and highlight where uncertainties or controversies currently exist. Furthermore, we outline major goals for future probe development and describe how progress in imaging methodologies will improve our ability to employ genetically encoded redox probes in a wide range of situations. This article is part of a special issue entitled "Redox Signalling in Heart."

Contents

1. Introduction

Redox signalling is increasingly recognised to play a vital role in physiology. Conversely, its dysregulation has been linked to numerous pathological processes, although cause-effect relationships often remain unclear. Cardiovascular physiology and pathology is no exception to this. On the one hand, it has become clear that physiological processes like cardiomyocyte differentiation and excitation–contraction coupling are under redox control. On the other, aberrant redox signalling seems to be associated with a variety of cardiac pathologies, including arrhythmia and myocardial ischemia-reperfusion [1–3]. Redox signalling is typically based on the specific and reversible oxido-reductive modification of particular cysteine residues in particular proteins. Redox-regulated proteins have been observed to partake in a wide range of cellular processes including signal transduction, gene expression and metabolism [4]. One specific example with relevance to cardiology is the ryanodine receptor 2 in which the redox state of cysteine residues determines Ca^{2+} conductivity and thus contributes to the regulation of the cardiac rhythm [2]. The key characteristic of redox signalling is that oxidative and reductive modifications are restricted both spatially and temporally. Under most circumstances, the generation, dispersal and elimination of superoxide and hydrogen peroxide is tightly controlled. Oxidative reactions are usually confined to specific subcellular compartments or microdomains, and the majority of oxidative and reductive processes are under kinetic control. Catalysts like NADPH oxidases (Nox), localised to specific cellular locations and activated at specific times, allow redox reactions to proceed in the direction of thermodynamic equilibrium only in a confined and transient manner. Elsewhere, kinetic barriers prevent uncontrolled equilibration between

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0022-2828/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2013.12.023

the many cellular redox species, at least on biologically relevant time scales. Cells frequently run both oxidative and reductive processes in parallel, even within the same compartment, relying on kinetic and steric separation to prevent thermodynamic equilibration. It therefore makes little sense to ascribe an overall redox state or redox potential to a cell. A redox potential can only be assigned to a chemically defined redox couple. Importantly, measuring one of these redox couples does not necessarily offer any information regarding the redox state of the other cellular redox couples. Thus, if one seeks to make a global statement about the "cellular redox status" or the "degree of oxidative stress," the measurement of different redox couples may well lead to very different conclusions. Moreover, the redox state of individual redox couples can differ widely between subcellular compartments, and whole cell measurements do not allow conclusions about individual compartments, e.g. the cytosol [5].

Taking all of the above considerations into account, it is clear that to better understand redox signalling and its dysregulation, we need to increase the resolution of our measurements. Resolution in this context means both spatiotemporal resolution and chemical resolution. It is imperative that we improve our ability to monitor specific, clearly defined redox species. We must improve our ability to monitor dynamic changes in these redox species with subcellular resolution. This will enable us to move away from poorly defined concepts that are frequently observed in the literature, such as cellular "reactive oxygen species (ROS)" changes or "oxidative stress."

The last 10 years have seen remarkable progress in our ability to address the issues of redox species specificity and spatiotemporal resolution. A particularly important contribution has been the development of genetically encoded redox probes, which now increasingly allow redox species-specific, dynamic real-time observations, with subcellular resolution in intact living cells, tissues and even whole organisms [6]. Arguably, these developments were initiated by a seminal publication of Jakob Winther and colleagues [7], describing the development of a redox-sensitive yellow fluorescent protein (rxYFP). This work triggered and inspired further improvements and developments that continue to expand our repertoire of genetically encoded redox probes to this day. We have now reached a stage where rapid redox changes can be observed in living mice, in real-time and with single-organelle resolution [8]. Although progress is obvious, a number of limitations, difficulties and uncertainties remain, and there is still much work to be done.

There have been several detailed reviews on the topic of genetically encoded redox probes, covering most of the relevant aspects, from chemical principles to practical applications [9–15]. It is not our intention to repeat what has been said already. Here we will discuss some questions relating to the specificity and sensitivity of current and future generations of genetically encoded redox probes. We highlight some of the problems that remain to be solved and offer suggestions as to how this may be achieved. Finally, we give a brief outlook on advancements that we may expect to see in the near future.

2. The question of specificity

A redox biosensor should be as specific as possible. Concerning specificity, it is important to distinguish two separate situations. In the first situation, the sensing redox pair, i.e., the redox probe, reversibly and dynamically equilibrates with a defined cellular redox pair, for example, GSSG/2GSH, and the redox interaction is clearly defined in both directions ($[Probe]_{RED} + GSSG \rightleftharpoons [Probe]_{OX} + 2\ GSH$). In this situation, perfect specificity would mean that the probe equilibrates only with the redox pair of interest and that there are no other (non-redox) influences that affect the probe response. In contrast, the situation is more complicated if the reaction of the probe with the redox species of interest is essentially irreversible, for example, the reaction of the redox probe with H_2O_2, for which the thermodynamic equilibrium lies almost completely on the side of the oxidised probe. In this case, subsequent probe reduction necessarily depends on interaction with another redox pair, for example, GSSG/2GSH. Strictly speaking, such probes are dual specificity probes, because they report on the interplay of an irreversible oxidative process and a separate reductive process. In principle, such probes respond not only to changes in the rate of oxidation but also to changes in the rate of reduction. These complexities may be considered a disadvantage; however, currently, there is no alternative if we want to make dynamic measurements of species like H_2O_2. On the other hand, this kind of probe behaviour may be seen as especially appropriate to study conditions of redox signalling because it closely mimics how many proteins are redox-regulated inside the cell (i.e., oxidation by H_2O_2, followed by reduction through the glutathione or thioredoxin system). Thus, ideally, probe specificity in this case would mean specificity for one defined oxidant and one defined reducing redox couple.

The development of genetically encoded redox probes was initiated with *de novo* engineering of redox sensitivity in proteins not normally redox-sensitive. The first such example was the rxYFP probe, wherein two cysteine residues were engineered to be present on the surface of the YFP β-barrel [7]. This was followed by the development of reduction–oxidation-sensitive green fluorescent proteins (roGFPs) [16,17], which are based on the same principle. In both proteins, the formation of a disulphide bond between the two cysteine residues changes the fluorescent properties of the protein, thereby allowing discrimination between the reduced and oxidised version of the protein. RoGFPs became more widely used than rxYFP, mainly because they allow ratiometric measurements and are not easily perturbed by pH changes.

From the very beginning, the question of specificity loomed large: which redox species are driving the oxidation and reduction of these reporters inside cells? It was soon established that both rxYFP and roGFPs equilibrate predominantly with the GSSG/2GSH redox couple, in a manner strictly dependent upon the presence of glutaredoxins, which catalyse the equilibration [18,19]. Their response therefore reflects changes in the glutathione redox potential (E_{GSH}), which is influenced not only by the GSSG:GSH ratio but also by the total glutathione concentration. The fact that both rxYFP and roGFPs are completely unresponsive to thioredoxin, most likely due to steric hindrances, contributes to specificity [9]. Concerning cardiovascular research, mitochondrially targeted roGFP has been expressed in cardiomyocytes to investigate the relationship between "ROS" and simulated ischemia-reperfusion [20]. However, it should be emphasised that rxYFP and roGFPs should not be called "ROS probes" because inside cells they are not direct targets of "ROS", even under rather extreme conditions [21].

Based on the above considerations, it is clear that probe specificity can be context-dependent. As roGFPs require the presence of a glutaredoxin to equilibrate with the GSSG/2GSH redox couple, it is unclear with what redox couple they will equilibrate within the endoplasmic reticulum (ER) where there are no glutaredoxins but instead a powerful protein disulfide generating machinery and multiple protein disulphide isomerases. Thus, it must not be assumed that roGFPs equilibrate with the GSSG/2GSH redox couple inside the ER. Further characterisation is needed to understand roGFP oxidation in that organelle. Of note, a strategy to enforce preferential equilibration of ER-targeted roGFP with GSSG/2GSH has been reported recently [64].

Other probes with engineered redox sensitivity have also been created, for example, Förster resonance energy transfer (FRET)-based probes, in which cysteine-containing peptides link two fluorescent proteins [22,23]. However, to our knowledge, it remains to be determined which redox species these probes respond to.

A second major advance in the development of genetically encoded redox sensors was the concept of employing proteins that naturally act as redox sensors. In principle, such probes are as specific as the natural sensors on which they are based. Typically, redox-sensitive protein domains are coupled to a reporter protein in a way that leads to a ratiometric change in fluorescence. There are two main ways to achieve this coupling. The first way involves the transmission of a

Please cite this article as: Ezeriņa D, et al, Imaging dynamic redox processes with genetically encoded probes, J Mol Cell Cardiol (2014), http://dx.doi.org/10.1016/j.yjmcc.2013.12.023