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Review

Using exomarkers to assess mitochondrial reactive species *in vivo* ☆,☆☆

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ABSTRACT

Background: The ability to measure the concentrations of small damaging and signalling molecules such as reactive oxygen species (ROS) *in vivo* is essential to understanding their biological roles. While a range of methods can be applied to *in vitro* systems, measuring the levels and relative changes in reactive species *in vivo* is challenging.
Scope of review: One approach towards achieving this goal is the use of exomarkers. In this, exogenous probe compounds are administered to the intact organism and are then transformed by the reactive molecules *in vivo* to produce a diagnostic exomarker. The exomarker and the precursor probe can be analysed *ex vivo* to infer the identity and amounts of the reactive species present *in vivo*. This is akin to the measurement of biomarkers produced by the interaction of reactive species with endogenous biomolecules.
Major conclusions and general significance: Our laboratories have developed mitochondria-targeted probes that generate exomarkers that can be analysed *ex vivo* by mass spectrometry to assess levels of reactive species within mitochondria *in vivo*. We have used one of these compounds, MitoB, to infer the levels of mitochondrial hydrogen peroxide within flies and mice. Here we describe the development of MitoB and expand on this example to discuss how better probes and exomarkers can be developed. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

Relatively short-lived, reactive molecules occur in many different contexts in biology [1,2]. Typical examples are the reactive oxygen species (ROS) superoxide and hydrogen peroxide [3,4], but there are many others including the reactive nitrogen species nitric oxide (NO) and peroxynitrite [5], degradation products arising from oxidative damage such as 4-hydroxynonenal (4-HNE) [6], and side products of metabolic processes, such as methylglyoxal from glycolysis [7]. These reactive species are of considerable interest because they can directly damage other biological molecules and disrupt cell function, and also they can act as signals to modulate biological processes [1,2,8,9]. Consequently many biologically important questions can only be addressed fully by measuring the steady state levels and changes in these reactive species *in vivo* [10,11].

Measurement of the concentrations of reactive molecules such as superoxide, hydrogen peroxide and NO in biological environments is inherently challenging due to their short lifetimes, diverse chemistries and the limited selectivity of detection systems [1,10,12]. It is possible to apply more selective approaches *in vitro* such as using spin trapping followed by electron paramagnetic resonance (EPR) spectroscopy to ensure that only radical species are detected, or by using catalase- or superoxide dismutase (SOD)-sensitive changes in the absorbance or fluorescence of detector molecules to provide robust and useful

Abbreviations: EPR, electron paramagnetic resonance; GFP, green fluorescent protein; 4-HNE, 4-hydroxynonenal; MitoB, 3-(dihydroxyboronyl)benzyltriphenylphosphonium bromide; MitoP, (3-hydroxybenzyl)triphenylphosphonium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; TPMP, methyltriphenylphosphonium; TPP, triphenylphosphonium cation

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information [12–14]. However, to address many biologically important questions it is often necessary to assess the level of the reactive species in context, and with these more complicated systems the detection methods are less selective and sensitive. In cell systems, general changes in the ROS can be detected by determining the oxidation of probes such as dichlorofluorescin to its fluorescent product dichlorofluorescein, although there are significant limitations to the use and interpretation of this probe [15]. The production of specific ROS such as superoxide can be inferred, with certain caveats, by determining the changes in fluorescence of probes such as hydroethidine [14] or MitoSOX [16], or for hydrogen peroxide by using boronic acid-conjugated fluorophores [17–19]. Another important approach is to utilise engineered proteins derived from green fluorescent protein (GFP) such as redox sensitive GFP (roGFP) or HyPer [20,21]. The fluorescent intensity of these probes responds to changes in endogenous redox couples or levels of hydrogen peroxide and often has the significant advantage that their fluorescence is ratiometric, thereby facilitating calibration, and that they can be directed to particular cell types and compartments [20–22]. These approaches work well and produce robust and useful information, provided artefactual effects are recognized and steps are taken to correct them [15,20,23]. However, extension of these approaches from cells in culture to intact living organisms is challenging. In some circumstances optical techniques can be used, for example in the optically accessible surface cell layers by the use of two photon microscopy [24], by the use of chemoselective bioluminescent probes [25], or in transparent organisms such as zebra fish embryos [26]. In general though, it is very difficult to measure directly the levels of small, reactive molecules within living organisms.

Changes in reactive species *in vivo* are often inferred by measuring the accumulation of oxidative damage markers derived from the interaction of reactive species with protein, DNA or lipid [27–32]. While measurements of oxidative damage markers are very informative, a major limitation is that the actual amount of accumulation of a damage marker is determined not only by its rate of formation but also by the effectiveness of repair processes and defence mechanisms, all of which vary independently of each other [30,33]. Another possibility is to measure the expression levels of genes that respond to alterations in a reactive species, either as a damage response or as a redox signal [34,35]. However, again there are multiple levels of regulation and feedback making the link between the expression level of the particular gene and the amount of a specific reactive species tenuous. The current situation is that while changes in the levels of short-lived reactive molecules are often proposed to mediate damage and redox signals in a range of biological situations *in vivo*, we do not have the techniques available to test these hypotheses properly [10]. There is a critical unmet need for better measurements of the levels of reactive species *in vivo*.

One approach that can be used to assess the levels and identities of reactive species *in vivo* is by using *exo*genous *marker* molecules for which we have coined the term "exomarkers". This approach has many parallels with the use of biomarkers whereby changes in the levels of products, such as F_2-isoprostanes, from the interaction of reactive species with endogenous molecules are used to infer changes in reactive species *in vivo* [32]. However, the use of exomarkers differs in that an exogenous, artificial probe compound is administered to the organism (Fig. 1). Within the organism the probe is modified by reactive species to generate an exomarker product which is diagnostic of the reactive species, and which then can be assessed and used to infer levels of reactive species present *in vivo* (Fig. 1). Despite previously lacking a unifying name, the concept of administering exogenous probe molecules to a living organism in order to infer changes in reactive species *in vivo* by the measurement of exomarkers is not new. It has been used by a number of other groups in the past. For example, the selective reactivity of spin traps with free radicals and the subsequent analysis of the products by EPR spectroscopy [36–38], or by a combination of liquid chromatography and mass spectrometry [39,40] has been extensively exploited within experimental animals. Similarly,

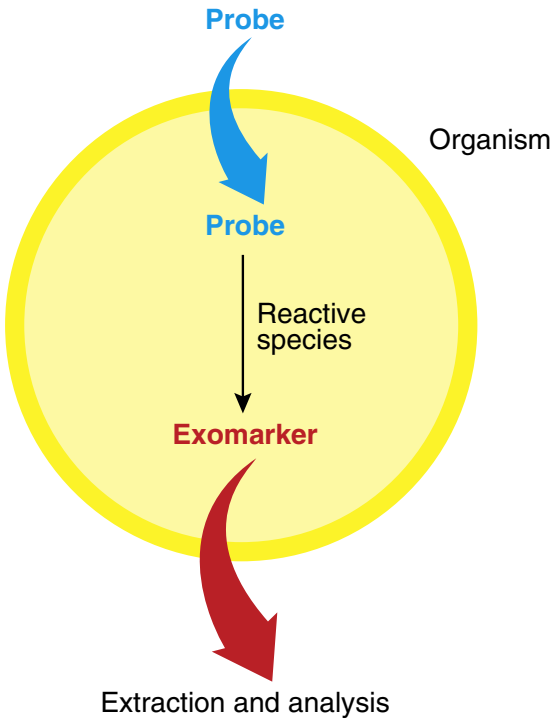

Fig. 1. The exomarker approach to assess levels of a reactive species *in vivo*. The probe is administered to the organism where it is converted to a diagnostic exomarker product by reaction with reactive species. The exomarker product is then extracted from the organism and analysed to infer the type and levels of reactive species that occur *in vivo*.

a range of chemical traps such as salicylate, DMSO, phenylalanine, 4-hydroxybenzoic acid and terephthalic acid have been used to infer the levels of hydroxyl radical production *in vivo* [12,41–47].

The ideal probe to generate an exomarker would have the following properties: non-toxic; does not alter endogenous metabolism; easily administered to the living organism; transports to a particular organ/tissue/cell type and localises within a single cell compartment; reacts selectively and efficiently with the target reactive species, with no side reactions, to produce a stable product; the reaction to generate the exomarker does not significantly alter the levels of the reactive species to be assessed; both the diagnostic exomarker and the probe are easily assessed at very low levels, ideally in urine or plasma. It is also important that the probe is synthetically accessible and chemically stable, and so an ideal probe is the simplest structure that fulfils all the requirements. While these are demanding criteria, many exomarker approaches will still be useful if they meet only some of them.

Here we describe our experience to date with one class of exomarker designed to report on mitochondrial ROS *in vivo* [11,48], discuss the strengths and weaknesses of this approach and consider how it may be extended and developed.

2. Assessing mitochondrial hydrogen peroxide *in vivo* with MitoB

In our laboratories one focus is to develop an understanding of the role of mitochondrial ROS such as hydrogen peroxide in biological damage and redox signalling [49–51]. The levels of mitochondrial hydrogen peroxide *in vivo* were not known, therefore to address this unmet need we developed an exomarker approach using a mitochondria-targeted mass spectrometric probe called MitoB [11,48]. MitoB (Fig. 2A) is comprised of the lipophilic cationic triphenylphosphonium (TPP) moiety linked to an arylboronic acid [11]. The TPP moiety has been used extensively to drive the uptake of a range of bioactive molecules into mitochondria *in vivo*, including antioxidants, probes and nitric oxide donors following oral, intravenous or intraperitoneal administration routes [52–55]. The lipophilic nature of the TPP moiety enables these molecules