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Moving Free Radical and Redox Biology Ahead in the Next Decade(s)

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Free Radical Biology; Redox Biology; reactive oxygen species; oxidative stress

The direct observation of free radicals in biological samples in 1954 provided support for what had been inferred for several decades, *i.e.* living, fully functioning organisms produce free radicals [1]. Following this, in conjunction with observations from radiation biology, Harman proposed the free radical theory of aging that is still being actively investigated [2]. The discovery of superoxide dismutase along with the realization that enzymes can make free radicals brought an entirely new view to the role of free radicals in biology [3]. Initially research focused on: the basic chemistry of the various free radicals and related oxidants; potential oxidative damage to cells and tissues; and the mechanisms and consequences that free radicals/oxidants could inflict in cells and tissues. However, this focus slowly changed as it was realized that superoxide and related oxidants in conjunction with superoxide dismutases are part of the basic biology of cells and tissues [4].

Because of the wide range of reactive species that could be generated in cells and tissues, the terms “reactive oxygen species (ROS)”, for the many oxidants, along with “oxidative stress”, to represent the consequence due to the damaging chemical reactions of these oxidants, were introduced in the 1980’s [5]; this vocabulary was a means to bring a more comprehensive and inclusive view to the field. However, these terms now appear to be a hindrance to the advancement of research in the field of free radical and redox biology because they provide very general information rather than the detailed information needed for deeper understanding.

For more rapid advances in the field of free radical and redox biology I suggest that investigators consider three matters:

1. vocabulary;
2. the information actually provided by instrumentation and associated chemical tools used in free radical research; as well as
3. quantitation.

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Vocabulary

The editors of *Free Radical Biology and Medicine* have published a Commentary offering a range of suggestions to improve the quality of research in free radical biology [6]. Included is the proposal that use of general terms such as ROS and RNS be minimized; such general terms should only be used when the actual species is unknown. The ubiquitous use of these terms seems to provide a screen to hide the detailed chemistry of these species; however, it is the unique chemistry of the individual oxidative species that needs to be appreciated and taken into account to allow the best interpretation of data and the best understanding of the free radical and redox biology being investigated. For example, the chemistry of singlet oxygen ($^1\text{O}_2$), superoxide ($\text{O}_2^{\bullet-}$), peroxy radicals ($\text{ROO}^\bullet$), and hypochlorous acid (HOCl) are very different, yet each is an ROS. If the species is known or strongly suspected, much information can be lost in communication if a general term, *e.g.* ROS, is used.

The Stress of Life, a classic book by Selye, laid the foundation for our thoughts on stress and health, from which our views on oxidative stress evolved [7]. The term *oxidative stress*, *i.e.* “a disturbance in the prooxidant-antioxidant balance in favor of the former” [5], has become widely used in redox biology. The vast majority of researchers associate the term with detrimental oxidative processes and consequences. However, I would posit that most of the changes in the oxidant-tone of cells and tissues are NOT detrimental; rather these changes lead to appropriate signals for adaptation to maintain optimal biochemical function and associated good health; see for example [8]. Goswami *et al.* have proposed that we use Selye’s term “eustress”, *i.e.* good stress, for oxidative events that regulate normal physiological functions, while “oxidative stress” be reserved for those settings where there are deleterious consequences from an oxidative event [9]. A more specific and better vocabulary conveys more information.

Chemical Tools and Instrumentation

As research in free radical biology moved from a focus on oxidative damage to the role of oxidants in normal, healthy biological settings many new tools have been introduced. The vast majority of analytical approaches rely on observation of changes in color. In free radical/redox biology this can be a color change, often a change in fluorescence, as a species is oxidized or reduced, such as the color change of an indicator molecule that is probing for changes in the levels of specific species, *e.g.* H_2O_2 , $\text{O}_2^{\bullet-}$, $\text{NO}^\bullet$. Prime examples are the many compounds that report on a chemical change after oxidation or reduction by an associated change in fluorescence [10]. The editors of *Free Radical Biology and Medicine* have strongly cautioned about the use and interpretation of data from experiments that use these tools [6, 11]. For example, 2,7-dichlorofluorescein diacetate (DCFH-DA) is widely used, and marketed, as a means to measure cell and tissue levels of H_2O_2 . After de-esterification intracellularly, DCFH can be oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF). Many researchers assume that the DCFH/DCF system reports on the intracellular levels of H_2O_2 . However, DCFH does not react directly with H_2O_2 [12]; rather H_2O_2 must first be “activated” by iron, *e.g.* iron as Fe^{2+} and the Fenton reaction or by reaction with Fe^{3+} -heme peroxidases. These reactions typically result in the one-electron oxidation of DCFH to a free radical, $\text{DCF}^{\bullet-}$. $\text{DCF}^{\bullet-}$ is a very reducing radical and will react

with dioxygen to form $O_2^{\bullet-}$ [13, 14], which with the aid of SOD will immediately dismutate to form H_2O_2 . The probe makes what it is alleged to measure; not a fruitful experiment with no clear interpretation.

Changes in pH can mislead researchers as well. For example, *para*-hydroxyl phenyl acetic acid and related phenols are also chemical tools used to measure H_2O_2 [15, 16]. However, careful attention to pH must be taken because the fluorescent dimer that reports on H_2O_2 has an apparent pK_a of 8.1 [15]. Thus, small pH differences in the samples of a set of experiments could be very misleading if not accounted for.

Another example is the circularly permuted yellow fluorescent protein (cpYFP). It has been used as an alleged reporter for superoxide production with claims that it can report transient changes in superoxide production, for example in mitochondria as “mitoflashes” [17]. However, a study using the xanthine/xanthine oxidase system as a source of superoxide could not demonstrate any reaction between $O_2^{\bullet-}$ and cpYFP [18]. With careful attention to controls it was concluded that the “mitoflash”-effect is most likely due to transient increases in pH in individual mitochondria. cpYFP has pK_a of ≈ 8.7 , thus small changes in pH can produce a substantial change in the fluorescent quantum yield.

These are only three examples where the properties and detailed chemical characteristics of the chemical tools must be understood by users to arrive at sound conclusions. There are many published papers that make conclusions from the response of chemical tools that are not appropriate. The data may not rule out the mechanism being proposed, but they cannot support the conclusion made. These types of reports hamper progress as they suggest directions that may be unfruitful, resulting in the loss of time and resources.

Quantitation in Redox Biology

The detection and quantitation of the different species that the term ROS encompasses is a challenge that has not yet been satisfactorily met. Kinetic modeling is an approach that can provide information on the levels, fates, and consequences of these species [19, 20, 21, 22]. Successful models require diffusion coefficients for those species that can move far from their origin, kinetic rate constants for the many possible reactions, and absolute quantitative information of the many enzymes, proteins, and reactive species involved. Unfortunately, most laboratory assays provide relative levels of species or enzyme activities in some arbitrarily defined unit. This information is not directly useful in modeling without a path to arrive at absolute levels. A goal in free radical and redox biology should be to evolve current assays and develop new approaches to arrive at absolute quantitative information that is directly comparable across laboratories, readily usable, and universally useful [16, 23, 24].

In addition to use in modeling, quantitation can provide context and crosschecks for data from different types of experiments. For example, there is great interest in cell and tissue production of H_2O_2 . It is clear that the rate of production of H_2O_2 will be less than the rate of oxygen consumption. Quantitation of these on an absolute basis using the same units provides investigators information on the validity of their H_2O_2 results. Unfortunately, there are published results that appear to show greater production of H_2O_2 than the O_2 consumed. Because the oxidation of H_2O is quite unlikely, such errors could be due to problems in data

analysis or, more troubling, a problem in the assay. Quantitative results expressed in standard, common units provides a common language and allows more in depth interpretations of data.

Addressing these three issues – *vocabulary*, *methods*, and *quantitation* -- will accelerate research progress and associated translation to improve the lives of all.

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