Free Radical Biology and Medicine 78 (2015) 233–235

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Even free radicals should follow some rules: A Guide to free radical research terminology and methodology

Henry Jay Forman [a,b,*], Ohara Augusto [c], Regina Brigelius-Flohe [d], Phyllis A. Dennery [e], Balaraman Kalyanaraman [f], Harry Ischiropoulos [g], Giovanni E. Mann [h], Rafael Radi [i], L. Jackson Roberts II [j], Jose Vina [k], Kelvin J.A. Davies [b,l]

[a] University of California at Merced, Merced, CA 95344, USA
[b] Ethel Percy Andrus Gerontology Center, Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA 90089, USA
[c] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil
[d] German Institute of Human Nutrition, Potsdam-Rehbrücke, Nuthetal, Germany
[e] Division of Neonatology, Department of Pediatrics, Children'sHospital of Philadelphia
[f] Department of Biophysics and Free Radical Research Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA
[g] Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania,Philadelphia, PA 19104, USA
[h] Cardiovascular Division, British Heart Foundation Centre of Research Excellence, King's College London, London SE1 9NH, UK
[i] Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay
[j] Department of Pharmacology, Vanderbilt University, Nashville, TN 37240, USA
[k] Department of Physiology, Faculty of Medicine, Universitat de València, Valencia, Spain
[l] Division of Molecular & Computational Biology, Department of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA 90089, USA

ARTICLE INFO

Available online 23 October 2014

Keywords:
Fluorescent dye
Reactive oxygen species
Reactive nitrogen species
Thiobarbituric acid
F2-isoprostane
Antioxidant
α-Tocopherol
Radical scavenging
Free radicals

ABSTRACT

Free radicals and oxidants are now implicated in physiological responses and in several diseases. Given the wide range of expertise of free radical researchers, application of the greater understanding of chemistry has not been uniformly applied to biological studies. We suggest that some widely used methodologies and terminologies hamper progress and need to be addressed. We make the case for abandonment and judicious use of several methods and terms and suggest practical and viable alternatives. These changes are suggested in four areas: use of fluorescent dyes to identify and quantify reactive species, methods for measurement of lipid peroxidation in complex biological systems, claims of antioxidants as radical scavengers, and use of the terms for reactive species.

Introduction—the problem and the need for some rules

The free radical field has undergone massive expansion in recent years. Emerging data indicate that the biological generation and reactivity of oxidants are harnessed to regulate numerous redox-dependent physiological processes. In turn, uncontrolled production and dysregulation of redox signaling are implicated in the initiation and propagation of several pathological conditions. Considering the vastly different backgrounds and training of free radical "practitioners," perhaps it is not surprising that agreement on common practices, including terminology, can be difficult.

Although we have no intent to impose restrictions on freedom of expression, we do suggest that some widely used practices are detrimental to progress in our field and need to be addressed. The goal of this paper is to identify such practices, make the case for their abandonment, and suggest practical and viable alternatives.

We have selected four areas that frequently cause concern and contention: (1) the use of fluorescent dyes to identify and quantify reactive species, (2) the thiobarbituric acid-reactive substances (TBARS) assay for lipid peroxidation in complex biological systems, (3) antioxidants as radical scavengers, and (4) recommended terminology.

Use of fluorescent dyes to measure reactive species

Although there are published methods that use the oxidation of fluorescent dyes to detect specific reactive species, misinterpretation of the data obtained from such dyes is a major problem in the

* Corresponding author. Fax: +1 (818) 301 4918.
E-mail address: peroxideman@gmail.com (H.J. Forman).

http://dx.doi.org/10.1016/j.freeradbiomed.2014.10.504
0891-5849/

free radical field. Recently, the editors of *Free Radical Biology & Medicine* addressed this issue [1] and made the following main recommendations:

(1) The reaction of reactive species with reporter dyes results in the generation of both specific, often less abundant, oxidized products and more abundant nonspecific products. Therefore the detection of a specific reactive species requires identification, separation, and quantification of the specific oxidation products. Furthermore, wise utilization of fluorescent dyes requires performing a series of controls in conjunction with molecular or pharmacological inhibitors for the identification of the reactive species involved.

(2) The most commonly used dye, dichlorodihydrofluorescein diacetate (DCFH-DA), is cell-permeative and undergoes intracellular hydrolysis to form the DCFH carboxylate anion, which is retained in the cell. Two-electron oxidation of DCFH results in the formation of the fluorescent product, dichlorofluorescein (DCF). However, DCFH does not react with superoxide, hydrogen peroxide, or nitric oxide. Instead, DCF fluorescence results from oxidation by potent oxidants, such those produced from metal ion- and peroxidase-catalyzed reactions and from proton- and carbon dioxide-catalyzed decomposition of peroxynitrite. Moreover, DCF-dependent fluorescence can be self-amplified by redox-cycling of the one-electron oxidized dye [2]. Possibly DCFH oxidation largely reflects the relocation of lysosomal iron to the cytosol [3] and peroxidase-catalyzed oxidation [4].

(3) Recommended sources for the measurement of reactive species include papers by Kalyanaraman et al. [1], Rhee et al. [5], Markvicheva et al. [6], and Van de Bittner et al. [7].

We suggest that the time has come for rigorous and precise use of these methods. We must stop accepting claims that reactive species are involved in a biological process based solely on the use of dye oxidation. It is essential that verification by separation of products or a more specific methodology be employed as suggested in the references cited above [1,5–7]. As such we recommend that scientific journals should not accept manuscripts containing improper interpretation of dye oxidation, and should request revisions. Provided that appropriate methodologies have been employed authors should be able to address the issue by appropriate revision of the text.

Thiobarbituric acid-reactive substances

In a test-tube experiment involving the oxidation of pure unsaturated fatty acids, thiobarbituric acid may be used to detect the formation of the lipid peroxidation product malonyldialdehyde (or malondialdehyde, or MDA) by production of a pink-colored product. In simple or highly purified systems, the TBA test for MDA gives an entirely appropriate estimate of lipid peroxidation.

Unfortunately, in more complex biological systems, many compounds (including simple and complex carbohydrates, protein oxidation products, and nucleic acid oxidation products) react with thiobarbituric acid to produce colored adducts. Thus, one cannot directly equate the measurement of TBARS with MDA or lipid peroxidation when measured in a complex biological system.

Therefore the use of TBARS as a sole indicator of lipid peroxidation in a complex biological system is not appropriate. In contrast, however, separation and mass spectroscopic analysis of thiobarbituric acid products, particularly MDA, have value in accessing the role of lipid peroxidation in oxidative stress as demonstrated by the multilaboratory investigation referred to as “BOSS II” [8].

We further suggest, however, that the best general indicator of nonenzymatic lipid peroxidation under normoxic conditions currently available is the measurement of F2-isoprostanes. These should be measured by mass spectrometry because the antibodies used in ELISA are not specific for F2-isoprotanes [9].

Antioxidants as scavengers of radicals and hydroperoxides

There are thousands of compounds that exhibit antioxidant chemistry in vitro and appear to have some “antioxidant effect” in vivo. Reactions of small organic compounds with most radicals are actually a competition in which the rate constants for the reactions are usually very close to one other. Thus, for a molecule to be effective as a scavenger, it would need to outcompete all other potentially reactive molecules present in the system. Only in cases in which an unusually high, localized concentration of an “antioxidant” molecule can be reached is this possible. An exception may be α-tocopherol. Based on consideration of its specific uptake and relatively rapid kinetics of reaction with lipid hydroperoxyl radicals ($LOO^{\bullet}$) compared with the propagation reaction ($LOO^{\bullet}$ + lipid), α-tocopherol may be an effective chain breaker in lipid peroxidation [10].

Scavenging of superoxide, hydrogen peroxide, and other hydroperoxides, all of which react relatively slowly with small organic molecules, is carried out efficiently by enzyme-catalyzed reactions that have rate constants that are 100,000 times faster than for their nonenzymatic counterparts. Thus, with the possible exception of α-tocopherol (and positively charged ubiquinone analogs that accumulate in mitochondria), physiologically meaningful scavenging by nonenzymatic reactions is essentially insignificant.Instead, many of the compounds referred to as “antioxidants” are most probably acting through their effects on signaling pathways, rather than reacting as true antioxidant scavengers. Please see Forman et al. [10] for a more complete discussion.

Hydroxyl radical scavenging

There are numerous misconceptions about so-called “hydroxyl radical scavenging” in biological systems by small molecules, including polyphenols, which have unfortunately resulted in many erroneous statements appearing in published papers. All organic compounds react with hydroxyl radicals with rate constants approaching the diffusion limitation. Thus, in solution, no compound really has any more significant hydroxyl radical scavenging activity than hundreds or thousands of other compounds (proteins, lipids, nucleic acids, amino acids, numerous metabolites, etc.) already present in any biological system. Therefore, for any compound to be even 50% effective in solution it would have to be present at a concentration equal to or greater than all of those other compounds together. Thus, there are no antioxidants for $^{\bullet}OH$.

Spin traps are often used as scavengers of hydroxyl radicals based on the formation of characteristic electron paramagnetic resonance (EPR)-detectable hydroxyl spin adducts. However, spin traps need to react with only a minute fraction of the total hydroxyl radicals generated in any given situation to yield a measurable EPR-sensitive radical adduct. Thus, although spin traps (e.g., DMPO) may not quantitatively inhibit hydroxyl radicals, they can still be used to detect hydroxyl radicals because of the high sensitivity and specificity of the EPR technique.

Thus, unless a molecule can convincingly be demonstrated to act in vivo as a direct scavenger of reactive species (as has been shown for α-tocopherol) we suggest that claiming such activity is no longer acceptable. This is not the same as showing that the molecule in question decreases the (damaging) effects of a reactive species, which may be through a mechanism different from direct scavenging. Additionally, limited intestinal absorption often significantly diminishes the effective concentration of an agent that can actually be achieved in vivo. We strongly encourage our colleagues to intensively investigate the mechanism(s) by which

their potentially effective "antioxidants" actually work in vivo, rather than just assuming that a test-tube antioxidant must also act as a scavenging antioxidant in vivo.

Demonstrating that an agent acts a scavenger when a reactive species is added to cells in culture alone is also not sufficient to identify a new physiological antioxidant scavenger, because the molecule in question may simply scavenge the reactive species outside the cells.

Although we feel that appropriately controlled studies of antioxidants in animals and humans are to be strongly encouraged, merely phenomenological accounts that do not rigorously investigate the mechanism(s) of action are strongly discouraged. Furthermore, if a study involves treatment of patients or animals with an antioxidant, it must follow the same rules that apply to studies of other potential pharmaceutical agents. In this regard, it must be shown that the dose of "antioxidant" tested actually lowered oxidative stress using quantitative methodology.

Recommended terminology: ROS, RNS, and other nonstandard abbreviations

We recommend that the abbreviations ROS and RNS not be used without definition. Winterbourn has discussed this issue [11] and concluded that, "The term ROS is generally taken to encompass the initial species generated by oxygen reduction (superoxide or hydrogen peroxide) as well as their secondary reactive products. Reactive nitrogen species (RNS) is also in common usage to describe reactive species derived from nitric oxide. There is clearly overlap and crosstalk between the production, function and decomposition of the two groupings, especially because of the highly favored reaction between superoxide and nitric oxide to give peroxynitrite."

Therefore we suggest that the preferred practice should be to use the name of the identified species. Of course we understand using the term ROS or RNS when the species is unknown or when it might be one of several molecules that have been implicated without certainty. But, it is then essential that this be clearly stated. The use of ROS and RNS in the text should be discouraged, particularly when the actual species are known or can be reasonably surmised. As an example, NOX (if defined as NADPH oxidase) may be used, but its products $O_2^{\bullet -}$ and/or H_2O_2, along with downstream products including hypochlorous and other hypohalous acids, should not be referred to as ROS. We suggest that ROS and RNS, as well as other nonstandard terms and abbreviations, should not be used in manuscript titles or in table or figure legends. The use of ROS or RNS in the text should be as stated above, i.e., only when it is clearly stated that the species is unknown or one of several implicated molecules without certainty.

Our goal here is not to reproach or reprimand anyone, but instead to contend that, at this point in the evolution of free radical biology and medicine, adherence to appropriate and accurate terminology and methodology is really needed to advance the field.

References

[1] Kalyanaraman, B.; Darley-Usmar, V.; Davies, K. J.; Dennery, P. A.; Forman, H. J.; Grisham, M. B.; Mann, G. E.; Moore, K.; Roberts 2nd L. J.; Ischiropoulos, H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic. Biol. Med.* **52:**1–6; 2012.

[2] Wardman, P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic. Biol. Med.* **43:**995–1022; 2007.

[3] Karlsson, M.; Kurz, T.; Brunk, U. T.; Nilsson, S. E.; Frennesson, C. I. What does the commonly used DCF test for oxidative stress really show? *Biochem. J.* **428:**183–190; 2010.

[4] Rota, C.; Fann, Y. C.; Mason, R. P. Phenoxyl free radical formation during the oxidation of the fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase: possible consequences for oxidative stress measurements. *J. Biol. Chem.* **274:**28161–28168; 1999.

[5] Rhee, S. G.; Chang, T. S.; Jeong, W.; Kang, D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. *Mol. Cells* **29:**539–549; 2010.

[6] Markvicheva, K. N.; Bilan, D. S.; Mishina, N. M.; Gorokhovatsky, A. Y.; Vinokurov, L. M.; Lukyanov, S.; Belousov, V. V. A genetically encoded sensor for H_2O_2 with expanded dynamic range. *Bioorg. Med. Chem.* **19:**1079–1084; 2011.

[7] Van de Bittner, G. C.; Dubikovskaya, E. A.; Bertozzi, C. R.; Chang, C. J. In vivo imaging of hydrogen peroxide production in a murine tumor model with a chemoselective bioluminescent reporter. *Proc. Natl. Acad. Sci. USA* **107:** 21316–21321; 2010.

[8] Kadiiska, M. B.; Gladen, B. C.; Baird, D. D.; Germolec, D.; Graham, L. B.; Parker, C. E.; Nyska, A.; Wachsman, J. T.; Ames, B. N.; Basu, S.; Brot, N.; Fitzgerald, G. A.; Floyd, R. A.; George, M.; Heinecke, J. W.; Hatch, G. E.; Hensley, K.; Lawson, J. A.; Marnett, L. J.; Morrow, J. D.; Murray, D. M.; Plastaras, J.; Roberts 2nd L. J.; Rokach, J.; Shigenaga, M. K.; Sohal, R. S.; Sun, J.; Tice, R. R.; Van Thiel, D. H.; Wellner, D.; Walter, P. B.; Tomer, K. B.; Mason, R. P.; Barrett, J. C. Biomarkers of oxidative stress study. II. Are oxidation products of lipids, proteins, and DNA markers of CCl_4 poisoning? *Free Radic. Biol. Med.* **38:**698–710; 2005.

[9] Milne, G. L.; Gao, B.; Terry, E. S.; Zackert, W. E.; Sanchez, S. C. Measurement of F2-isoprostanes and isofurans using gas chromatography–mass spectrometry. *Free Radic. Biol. Med.* **59:**36–44; 2013.

[10] Forman, H. J.; Davies, K. J.; Ursini, F. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. *Free Radic. Biol. Med.* **66:**24–35; 2014.

[11] Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* **4:**278–286; 2008.