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Redox Regulation Beyond ROS

Why ROS Should Not Be Measured as Often

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Based on the concept of oxidative stress, reactive oxygen species (ROS) have been incriminated as the drivers behind almost every cardiovascular pathology. Redox alterations are, however, omnipresent bystanders to changes in cellular activity state. Even when ROS levels are altered, their contribution to pathology is not necessarily causal. Researchers should hesitate to engage in global ROS measurements and rather aim on identifying individual molecular targets of redox regulation.

The search phrase (ROS or reactive oxygen species) retrieves >132 000 hits in Pubmed. When restricted to the publication date of 2017, the search query returns 18 hits for *Circulation Research*. Essentially, the majority of ROS studies follow a similar mechanistic pattern: cells, tissues, or whole animals are subjected to a stress challenge (ie, a disease model), and ROS are measured and reported to be increased. In more appropriate studies, like those appearing in *Circulation Research*, molecular targets of ROS are identified (ie, oxidation of individual proteins) in signaling cascades. Subsequently, the system is subjected to interference (for example, siRNA or an inhibitor), which prevents the stress-induced phenotype, reduces ROS level, and alters target-protein oxidation. On this basis, it is concluded that ROS mediate the biological effects of the stressors. Although this study design is mechanistic and thus attractive, some particular aspects of redox biology should be taken into account to avoid overinterpretation of the findings.

ROS Changes Are Common

Are you aware of many ROS studies reporting changes in the activity state of cells, tissues, or organs without a change in ROS level? Certainly, the question of ROS involvement already imposes a bias toward altered ROS level, but it also seems that most physicochemical stimuli, that is, flow, stretch, low Po_2, high Po_2, changes in pH and numerous growth factors, hormones, and cytokines, alter ROS. Moreover, in all common cardiovascular disease models, ROS levels are increased.[1] Why is this the case? The actual ROS level depends on numerous factors: activity of ROS-producing systems, activity and capacity of the thiol antioxidant system, oxygen tension, and numerous other elements like metabolic supply. Studies on the thiol redoxome documented that changes in ROS level impact differentially on numerous thiols.[2] Conversely, changes in the redox state of thiols impacts on cellular function and can stimulate ROS production.[3] Thus, ROS and redox biology are tightly interlinked to form a complex analog redox network. ROS are, therefore, surrogate indicators of the redox network; changes to the network alter ROS and vice versa. It even seems that evolution has captured the oxygen stress response for signaling purpose toward inflammation. In cell culture models, hyperoxia, hydrogen peroxide, and a broad array of stimuli including tumor necrosis factor α, angiotensin II, thrombin, or interleukin 4, just to name a few, activate pathways resulting in antioxidative and proinflammatory responses.[1] Nevertheless, this antioxidative response is not harmful but rather represents general signaling paradigms, such as the following: The use of ROS to inactivate phosphatases for the facilitation of receptor-mediated signaling.[4] Importantly, this mechanism is even required for bona fide beneficial stimuli, like VEGF (vascular endothelial growth factor).[1]

The Term ROS Is Too Broad

Anything that contains oxygen and is at least slightly reactive belongs to the group of ROS. The broad term, however, ignores that the biology between the individual types of ROS varies tremendously. Hydroxyl radicals are the extreme of highly reactive ROS, which are so aggressive (and thus short-lived) that they react on diffusion limit with any molecule. Thus, hydroxyl radicals are primarily unspecific toxins and do not serve a signaling function as this would require a specific preference toward a molecular target. As a side note, this also implies that hydroxyl radicals cannot be measured quantitatively in biological systems as they have a low probability of reacting with ROS tracers.

Dioxygen, the normal atmospheric molecular form of oxygen, is the other extreme type of ROS. Because of its diradical nature (2 unpaired electrons), oxygen is also reactive, but the reaction speed is so low that it takes days for some lipids, or years for DNA, to oxidize. Also the signaling relevant types of ROS (nitric oxide [NO], $ONOO^-$, H_2O_2, O_2^-, and lipid peroxides) have vastly different molecular targets and levels of reactivity.[1] Because of the complex reactions among these

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The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

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(*Circ Res*. 2018;123:326-328.
DOI: 10.1161/CIRCRESAHA.118.313146.)

***Circulation Research* is available at http://circres.ahajournals.org**
DOI: 10.1161/CIRCRESAHA.118.313146

molecules, it is often difficult, if not impossible, to deconvolute the biological function of a complex ROS signal or to identify the specific type of ROS responsible for the signaling response. This, however, is important as our understanding of redox biology is far from complete. The interaction of O_2^- with NO and its important biological consequences, like endothelial dysfunction, can be considered as textbook knowledge.[1] However, much less is known concerning the molecular targets, even those of NO.[5] Moreover, O_2^-, NO, and H_2O_2 have gained more attention than other types of ROS that could be equally important yet less frequently studied. In particular, lipid peroxides have numerous biological actions ranging from thiol oxidation[6] to receptor-mediated signaling and the formation of secondary lipid peroxides from auto-oxidative chain reactions in an oxygen containing environment. In fact, we are only beginning to understand the biological functions of these molecules. Because many popular ROS assays do not discriminate between those types of ROS,[7] they impose a strong bias toward popular molecules.

ROS Assays Are Trimmed for Positive Results but Not for Accuracy and Specificity

The methodology to measure ROS is constantly improving, but particularly in the clinical setting the current tool box is, however, still suboptimal. Although novel ROS tracers are continuously being developed, some general chemical problems render ROS measurements difficult. Applications in intact biological samples are challenging as the ROS tracer has to outcompete other reaction partners of ROS to yield a signal. This is difficult to achieve, particularly if the tracer concentration must be kept low, not to interfere with cellular function. Therefore, ROS measurements in biological systems do not provide absolute quantification of ROS level. Low assay signals should also be expected for most situations. In search for ideal reagents, a large number of ROS assays have been developed.[7] To generate a strong signal, some of the more popular reagents, like dihydrodichloro-fluorescin, increase the ROS signal by auto-oxidation, amplification, or redox cycling. Menadione, NADH, NADPH, peroxidases, EDTA, vitamin C, luminol, lucigenin, coumarins, flavins, and ubiquinones are all compounds that chemically convert a reductive or oxidative signal into a strong unspecific ROS signal, which can be detected by many standard assays. Thus, results of these assays should be considered only as indicators of altered ROS level, rather than absolute quantitative values. Most assays are also not specific for ROS. For example, the standard assays for O_2^- have difficulties in discriminating between O_2^--mediated reduction and direct enzymatic reduction.[7] The diaphorase activity of the NO synthase in the nitroblue tetrazolium assay is one example of such a false-positive reaction.[7]

H_2O_2 is less reactive than most ROS and therefore harder to detect. Standard H_2O_2 assays add peroxidases to the reactive tracer, which converts H_2O_2 into highly reactive intermediates. This trick is also the basis for the frequently (mis-)used and heavily criticized dihydrodichloro-fluorescin assay,[7,8] which requires intracellular peroxidase activity. The inclusion of peroxidases in the assay is problematic for several reasons: Changes in peroxidase activity could give the false impression of an altered ROS level; peroxidases in the presence of reducing equivalents generate ROS and ROS signals themselves and, during inhibitor screens, peroxidase inhibition results in false-positive hits. Of the established chemical assay systems, boronates are the only peroxidase-independent probes for H_2O_2. Although some interesting probes have been developed to allow for H_2O_2 measurements directly in mitochondria,[9] the suitability of boronates to detect total cellular H_2O_2 or H_2O_2 release has been questioned. This is because of its high reactivity with peroxynitrite and because the H_2O_2 signal generated by Nox-NADPH oxidase overexpressing cells is low. These limitations restrict the use of boronates in whole-cell measurements to inflammatory cells and overexpression systems.[10]

Novel ROS sensors are continuously being developed. In particular, the inclusion of mass spectrometry to specifically determine reaction products of the probes with different types of ROS resulted in a gain in specificity and sensitivity.[11] The disadvantage of these newer probes, however, is that they are unsuitable for online measurements. To overcome this problem, and as an alternative to chemical ROS tracers, protein-coded fluorescent ROS sensors have been developed.[12] Although these are generously distributed by their creators, few publications report the use of these tools. This might be for several reasons. First of all, the systems are more difficult to handle than chemical tracers. Cells have to be transfected, and measurements are technically more demanding and often ratiometric. The assays are also subject to photobleaching, phototoxicity, and other artefacts (eg, movement of the sample and pH changes). Even more importantly, the dynamic range of the assay and the signal to noise ratio of the fluorescence signal, and thus their sensitivity is often low. Using protein-coded fluorescent redox-sensors, we failed to observe an acute growth factor-dependent increase in cellular ROS level. Obviously, we cannot exclude that, in addition to the low sensitivity, inadequate handling might have been responsible for this failure. An alternative interpretation could be, that, because of the problems of the traditional sensors, false-positive results were obtained, which do not hold true when re-evaluated with more up-to-date methodology.

The lucigenin assay in cellular homogenates is one such example. In principle, a cell or tissue homogenate is supplemented with NADH or NADPH, and subsequently ROS formation is measured with a ROS tracer, usually lucigenin. Although the assay was instrumental to kick-off research on Nox-family NADPH oxidases, it is now clear that the initial findings were not a consequence of O_2^- formation but rather of redox-cycling,[7] and there remains some controversy on the enzymatic system detected by the assay.[8,13] It is unclear why the signal in the assay is only partially sensitive to highly potent Nox inhibitors or to superoxide dismutase. Why overexpression of Nox enzymes increases ROS signaling in living cells by several orders of magnitude yet hardly changes the activity in the lucigenin assay remains a mystery.[13] Other examples of problematic assays are inhibitor screens using peroxidase-coupled reactions, dihydroethidium applied to frozen samples,[7] the amplex red assay in isolated mitochondria, and discrimination of ROS generators on the basis of unspecific compounds, like diphenylene iodonium, apocynin, or N-acetylcysteine.

Clinical Confirmation of Antioxidant Therapy Is Pending

Despite a large body of literature linking oxidative stress to diseases, prospective intervention trials on antioxidants were essentially negative. It is with good reason that the American Heart Association issued "….there is still no conclusive evidence that ROS/RNS are fundamentally involved in the pathogenesis of cardiovascular disease in humans…".[7] An attractive explanation for this is that any interference with the complex redox network will result in compensatory changes, thereby neutralizing the interference. Another conclusion could be that altered ROS and oxidative stress are epiphenomena and bystanders or permissive factors of signaling processes. Both considerations not only explain the failure of antioxidant therapies but also emphasize that measurements of ROS will always reveal transient alterations in ROS level. These findings will probably not be specific or give much direction. Rather, specific targets of individual ROS entities and unitary pathways of redox signaling have to be identified to foster the development of a specific therapy.[1]

Stop Measuring ROS

The general conclusion will likely be that ROS are altered in pathology and signaling anyway and that the determination of ROS is often dispensable. It should be noted, however, that not all reviewers of this text agreed on this matter. Failure to detect increased ROS level does not exclude subdomain ROS alterations and might even give rise to controversies distracting from the true biological process. On the contrary, increased ROS levels will not reveal the individual type of ROS mediating the effect. Most importantly, the mechanistic part in a redox-study has to be the identification of the molecular ROS target. This task, however, is methodologically distinct from ROS measurements, and ROS measurements will not help in target identification. Moreover, the interplay between redox chemistry (iron oxidation, antioxidant enzymes, and their cofactors) and ROS is so complex that it cannot be derived from ROS measurements. With the rapid progress in redox proteomics taking advantage of redox labels in combination with mass spectrometry identification of molecular targets of ROS and discovery of novel mechanisms of ROS signaling have become more feasible and accessible. With the aid of knock-in strategies in transgenic mice for individual redox-sensitive cysteins, it has become possible to describe the in vivo function of individual redox modifications and their disease relevance.[14] These approaches may eventually lead to a site-directed redox therapy, which could be more specific and thus potentially more successful than the antioxidant approach to cardiovascular disease.

In conclusion, ROS measurements are problematic and often dispensable for up-to-date mechanistic studies. Researchers should consider these aspects before engaging in ROS measurements.

Sources of Funding

This work was supported by the Goethe-University, the Heinrich und Fritz-Riese-Stiftung, and the Deutsche Forschungsgemeinschaft DFG (SCHR 1241/1-1, SFB 815 & SFB 834).

Disclosures

None.

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KEY WORDS: antioxidants ■ cardiovascular diseases ■ cytokines ■ oxygen ■ reactive oxygen species