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Unravelling the Biological Roles of Reactive Oxygen Species

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Summary

Reactive oxygen species are not only harmful agents that cause oxidative damage in pathologies, they also have important roles as regulatory agents in a range of biological phenomena. The relatively recent development of this more nuanced view presents a challenge to the biomedical research community on how best to assess the significance of reactive oxygen species and oxidative damage in biological systems. Considerable progress is being made in addressing these issues and here we survey some recent developments for those contemplating research in this area.

Keywords

Reactive oxygen species; oxidative damage; redox signalling; aging; antioxidants

Introduction

Reactive Oxygen Species (ROS), oxidative stress and oxidative damage are increasingly assigned important roles in biomedical science as deleterious factors in pathologies and

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aging. There is also the growing recognition that many ROS are in addition important mediators in a range of biological processes such as signalling. However, this greater interest in ROS raises the concern that too often a certain biological phenomenon is ascribed to ROS or oxidative damage based on inadequate rationales, technical approaches or understanding of what is chemically plausible. This tendency is surprising as there is considerable knowledge available on the detailed chemistry of individual ROS and the oxidative reactions that can occur within biological systems. However, this knowledge is often seen as technically specialised or inaccessible to those in other areas of biomedical science whose research, perhaps unexpectedly, leads them to suspect a role for ROS in their work. Consequently, there are many examples of otherwise well-conducted studies of considerable general interest that contain superficial or flawed conclusions about the involvement of ROS in the process investigated. A corollary is that technical approaches to measuring and blocking the actions of ROS and oxidative damage within biological systems are often difficult to interpret and prone to artifact. Consequently results need to be assessed cautiously with a clear understanding of what the methods used do or do not measure. The multiple facets of this problem pose a challenge to those studying the chemical and biophysical sides of ROS to explain better what is possible and what is not, and to develop and publicise more effective tools for investigating the impact of different ROS, particularly *in vivo*.

To discuss current understanding of ROS in biology, and to explore how challenges in the field could be addressed, a group of leading researchers with biological and chemical perspectives on ROS was brought together for an interdisciplinary conference, “The Chemistry and Biology of Reactive Oxygen Species”, funded by the Wenner-Gren Foundations and held in Stockholm from September 8-11, 2010. A number of common threads emerged from the meeting that are of general interest to the biomedical research community.

Making the chemistry explicit

A major recurrent theme from those working on the chemistry of ROS and oxidative damage was the importance of understanding the nature of the particular ROS under consideration in a biological context (Winterbourn, 2008). In other words, “ROS” is often used imprecisely in the biomedical literature as a monolithic term, as if all ROS molecules were the same. Of course, there are many situations in which the use of ROS as a generic description is appropriate. However in some cases it can be unhelpful and misleading because biologically important ROS encompass a diverse range of species, including superoxide, hydrogen peroxide, nitric oxide, peroxynitrite, hypochlorous acid, singlet oxygen and the hydroxyl radical. Each of these molecules is a distinct chemical entity with its own reaction preferences, kinetics, rate and site of production and degradation and diffusion characteristics in biological systems. Consequently the biological impacts of “ROS” depend critically on the particular molecule(s) involved, and on the microenvironment and physiological or pathological context in which it is being generated. To group all ROS together as a single entity is imprecise and can lead to vague and untestable hypotheses. Therefore, wherever possible the particular species thought to be responsible for the phenomenon under study should be specified.

A similar error is a tendency to treat all antioxidants as if they were alike, when in fact each has its own chemical properties and selective reactivity with particular types of ROS, as well as distinctive distribution, metabolism, recycling and other potential effects within a biological context. For example, Vitamin E and Vitamin C interact with different ROS *in vivo* in quite different ways because of their very different chemistry and in addition because the former is present within lipids while the latter is found in the aqueous phase. Furthermore, neither they nor most other small molecule antioxidants react with hydrogen peroxide. Enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, catalase or peroxiredoxins are selective for particular ROS and some, such as superoxide dismutase, act by converting one type of ROS (superoxide) into another (hydrogen peroxide) and by preventing formation of a further potent oxidant (peroxynitrite). Thus an antioxidant may block the activity of one type of ROS but leave another form unscathed. Furthermore, localization and catalytic activities of antioxidant enzymes may be regulated via post-translational modification (such as acetylation, phosphorylation, cysteine oxidation) and interaction with other proteins. For example, the mechanisms by which peroxiredoxins remove peroxides and peroxynitrite require disulfide reduction by thioredoxin. Moreover, mammalian thioredoxins are themselves regulated by reversible inactivation via two-disulfide formation or nitrosylation involving structural cysteine residues (Hashemy and Holmgren, 2008). Such regulation may provide a means of generating favorable gradients of hydrogen peroxide around the appropriate location at the right time for signaling in response to stimulation of cell surface receptors (Woo et al., 2010). In addition, aquaporins and related channels may provide a mechanism to regulate local hydrogen peroxide fluxes (Miller et al., 2010).

In summary, it would be helpful if researchers specified the particular ROS and the reactions thought to be responsible for given biological effects, and wherever possible and appropriate, avoided vague usage of generic terms like ROS and antioxidant. Likewise, researchers should show that an antioxidant actually reacts with the particular ROS under discussion and can, at least in principle, lower the concentration of that ROS sufficiently *in vivo* to explain its action.

Determining if a particular ROS plays a role in a biological phenomenon

To overcome the shortcomings outlined above, we recommend that researchers (and reviewers) should routinely go through a checklist of a few simple, common sense principles before assigning a proposed redox mode of action of ROS and antioxidants (Table 1). This has been suggested before (Gutteridge and Halliwell, 2010; Halliwell and Gutteridge, 2007), and is, if you like, equivalent to “Koch’s postulates” for ROS and oxidative damage. It will not be possible to satisfy all these criteria in every case, for example the direct measurements of particular ROS suggested in Table 1 may not be feasible *in vivo*. Even so, these criteria are still useful to assess whether the conclusion reached or the hypothesis proposed is in principle plausible. Without such a heuristic approach it is difficult to determine if a change in concentration of a particular ROS or the effect of a certain antioxidant is indicative of a role for a specific ROS in a pathology or redox signal. Alternative interpretations should be considered, for example oxidative damage can accumulate as a secondary consequence of other events (e.g. changes in repair rates, since

oxidative damage and its repair occur continually in aerobes) and many of the molecules used as antioxidants have other pharmacological effects.

The above considerations can help identify shortcomings in ROS-related investigations, some of which occur regularly in the biomedical literature. For example, there is a tendency to automatically link mitochondrial dysfunction with increased production of superoxide or hydrogen peroxide. However, the experimental support for this link is weak as there are many examples of mouse models with severe respiratory chain dysfunction that do not have any major increase in hydrogen peroxide or superoxide production or oxidative damage (Wang et al., 2001; Trifunovic et al., 2005; Kujoth et al., 2005). In addition, studies of isolated mitochondria suggest that the link between hydrogen peroxide or superoxide production and mitochondrial dysfunction is complex (Murphy, 2009). Finally, it should be mentioned that the role for oxidative damage in aging as originally proposed (Harman, 1956) is still actively debated. For example, experimental studies imply that moderately increased mitochondrial oxidative damage is well tolerated and does not affect life span in the mouse (Jang and Van Remmen, 2009).

Another area that can be problematic is interpreting the effects of antioxidants. Consider the use of superoxide dismutase (SOD) mimetics to test whether superoxide is responsible for a particular effect in a biological system. The rate constants for SOD mimetics are generally 100-fold lower than that of enzymatic SOD itself, which is often present at concentrations greater than 10 μM *in vivo*. Consequently it is important to show that the SOD mimetic increases tissue SOD activity significantly over endogenous levels (e.g. Keaney et al., 2004) before concluding that its biological activity is due to depleting superoxide. Another consideration is that it is most unlikely that the biological mode of action of any antioxidant is through hydroxyl radical scavenging (Halliwell and Whiteman, 2004). This is because the hydroxyl radical reacts at a diffusion limited rate with most biological molecules, so no exogenous molecule could achieve the concentration required to compete with this process (Halliwell and Whiteman, 2004). A further point is the widespread use of N-acetylcysteine (NAC) as a generic antioxidant in biological situations (Atkuri et al., 2007). As a membrane permeant cysteine precursor, many of its effects may be due to increasing cellular thiol (e.g. GSH) levels, but often this is not examined and many other effects may contribute (Zafarullah et al., 2003). For example, an increase in the free thiol content within the cell, or perhaps just in the cell culture medium (Xu and Thornalley, 2001; Mi, et al, 2010), may explain many of the observed effects. However, direct scavenging of hydrogen peroxide by NAC is unlikely as the reaction between these species is very slow. In addition, NAC might exert its effect by causing structural changes in cell surface proteins (Hayakawa et al., 2003), because the extracellular domain of many cell surface receptors contains disulfide bridges, which can be reduced by NAC (Hayakawa et al., 2003). Thus, the action of NAC may be more as a redox modulator and it should not be described as an antioxidant unless there is specific evidence that it is acting in this way. Similarly, many other compounds such as dietary polyphenolics act as antioxidants in simple *in vitro* systems but it is often unclear if the compound is present at a sufficient concentration *in vivo* to lower the levels of the relevant ROS. Consequently, many “antioxidant” effects *in vivo* may be due to some other pharmacological interaction distinct from a decrease in a particular ROS. Therefore,

proposed lowering effects of specified ROS by a particular antioxidant should, if possible, be confirmed by measuring levels of the particular ROS supposedly scavenged by the compound.

Measuring ROS production and oxidative damage

A major theme that emerged from the meeting is the urgent need for better approaches to measuring the different types of ROS and forms of oxidative damage that occur in biological systems, particularly *in vivo*. It is important to distinguish between the measurement of particular ROS themselves and the assessment of the damage that these ROS cause. Biomarkers of oxidative damage such as protein carbonyls, lipid peroxidation products or breakdown products of damaged DNA are often used, particularly *in vivo*, to infer production of ROS such as the hydroxyl radical. However the link between the accumulation of a marker for oxidative damage and production of a particular ROS is indirect, because a change in clearance can dramatically alter the level of the marker with no change in production of a given ROS. For example protein carbonylation is elevated by mistranslation of proteins, even when the levels of ROS are unaltered, due to an increased susceptibility of misfolded proteins to oxidative attack (Dukan et al., 2000). Conversely, as hydrogen peroxide can act as a redox signal without causing significant oxidative damage, its levels can change in biologically significant ways without necessarily resulting in oxidative damage accumulation.

Validation of better biomarkers for the assessment of oxidative damage *in vivo*, particularly in human samples, should be a priority. A great deal of research effort is directed toward linking the detailed chemistry of oxidative damage to protein, lipid and DNA with the production of measurable biomarkers (Jacobson et al., 2010; Portero-Otin et al., 2004). The current best available biomarker for lipid peroxidation seems to be isoprostanes. However work (e.g. Xue et al., 2009) on the detailed measurement of oxidative damage to proteins and nucleic acids by mass spectrometry suggest that many more biomarkers are likely to be developed and our understanding of the link between the levels of biomarkers such as protein carbonyls, 8-hydroxydeoxyguanosine, F_2-isoprostanes and reactive aldehydes that can be measured in biological fluids, and the reactions that lead to their accumulation may assist this. A corollary of this is that it is unlikely that a single biomarker will ever give a complete picture of oxidative damage *in vivo* as different types of damage to lipids, the proteome or the genome will lead to distinct patterns of accumulation of biomarkers, while interventions such as antioxidants will also affect the accumulation of biomarkers differentially. Hence whatever biomarkers are used they are not necessarily a simple single marker of overall oxidative damage but will each respond differently to alternative types of damage and interventions.

Research into new approaches in the technically difficult area of measuring the levels of specific ROS in biological systems is gaining momentum. Often these experiments are done with redox-sensitive probes that are very susceptible to artifactual side reactions. For example, dichlorodihydrofluorescein (DCFH) oxidation to the fluorescent dichlorofluorescein (DCF) is widely employed and is ofen described as measuring ROS production. It is frequently inferred that this is synonymous with hydrogen peroxide

production, but all too often evidence for this link is not made. Furthermore, there are many issues with respect to the specificity of this assay, as discussed in more detail elsewhere (Wardman 2007). A major consideration is that DCFH does not react directly with hydrogen peroxide but requires a peroxidase or transition metal catalyst. The signal also depends on the extent of uptake of DCFH, so there are many ways in which it can vary without any change in hydrogen peroxide production (Wardman, 2007). In addition, DCFH is readily photosensitized and can also catalyze the production of superoxide. Finally, the relationship between the intensity of DCF fluorescence and the concentration of the ROS under consideration is often assumed to be linear, in that a doubling of fluorescence indicates a doubling of the ROS concentration, however the relationship between DCF fluorescence and particular ROS levels is often non-linear (Wang and Joseph, 1999), perhaps due to changes in levels of transition metals that convert more H_2O_2 into hydroxyl radical or to the artifactual generation of superoxide and hydrogen peroxide by this probe (Halliwell & Gutteridge, 2007; Wardman 2007). Caveats also apply to the interpretation of results obtained with other probes such as hydroethidine, its mitochondria-targeted derivative MitoSOX, and Amplex Red which also have side reactions and a range of associated artifacts (Zhao et al, 2005; Selivanov et al., 2008). There is no simple solution to the difficulty of measuring a particular ROS accurately within biological systems but a more cautious use of probes and an effort to corroborate the measurement using orthogonal techniques is needed.

In developing the next generation of probes for specific ROS a few simple principles must be observed: the location of the probe molecule within the cell or organism must be understood; the chemistry and rates of the reaction of the probe with different types of ROS must be clear so that the particular ROS responsible for the response can be established; and the subsequent metabolism or decomposition of the oxidised probe must be understood (Zhao et al, 2005; Winterbourn, 2008). In doing so, the parallel assessment of the probes to correlate the end points of their reactions with the ROS of interest to the changes in signals such as fluorescence and an assessment of the sensitivity of the probe to particular ROS, as has been done to characterise the superoxide-selective hydroethidine probe (Zhao et al., 2005; Zielonka et al, 2008), will be essential. Using these approaches to measure particular ROS in simple systems such as isolated enzymes or mitochondria helps to better understand the basic processes involved. However these findings should be extrapolated to the *in vivo* situation with great caution, if at all, because often the factors determining the production of a particular ROS *in vivo* are quite different from those *in vitro* (Murphy, 2009). Therefore there is a great need for methods to measure ROS *in vivo* and considerable progress has been made with probe development for live imaging, although some of this is still in the chemical literature and has yet to be widely adopted by the biomedical community.

One increasingly important approach is through the engineering of green fluorescent protein (GFP) and its variants to produce redox-sensitive fluorescent probes, such as roGFP (Hanson et al., 2004) and HyPER (Belousov et al., 2006). These proteins incorporate redox-sensitive cysteines into the β-barrel of GFP (Hanson et al., 2004), or integrate GFP and related derivatives into redox-regulated protein motifs, such as the hydrogen peroxide sensor from OxyR (Belousov et al., 2006) or the yeast peroxiredoxin Orp1 (Gutscher et al., 2009).

These procedures generate probes that react selectively with a particular ROS such as hydrogen peroxide and then undergo changes in fluorescence with a high degree of sensitivity. There are a number of advantages of these fluorescent protein probes: the signal is reversible, allowing its calibration in the cell thereby permitting a comparison of basal levels of particular ROS or the status of particular redox couples among different cell types, or in response to various stimuli, something that is not possible with probes such as DCFH (Meyer and Dick, 2010). Another important advantage is that these proteins can be easily modified with targeting sequences so as to be expressed at particular intracellular locations such as the mitochondria or endoplasmic reticulum and thereby report on localised redox changes within the cell (Enyedi et al., 2010). Finally, using cell-specific promoters, these sensors can be introduced into the genome to generate transgenic animals that demonstrate cell- and region-specific expression of the sensor (Guzman et al., 2010). Such methods can be readily translated to *in vivo* models in transgenic animals expressing these redox sensitive fluorescent proteins, such as zebrafish or worms, or in conjunction with two photon confocal microscopy techniques to probe deeper beneath the surface in non-transparent animals. These methods can also be combined to study the redox changes within organelles in specific cell types, for example by using a tyrosine hydroxylase-promoter-driven mitochondrial roGFP it was possible to explore how DJ-1 affects mitochondrial redox state within dopaminergic neurons (Guzman et al., 2010). Finally, the mid-point potential of the roGFP sensors can be manipulated by inserting an extra amino acid between the cysteine residues in the sensor, making it more difficult to oxidize. This approach has already been reported to be effective in assessing the redox state of the endoplasmic reticulum, where the more oxidizing environment renders the original sensor ineffective because it remains almost fully oxidized under basal conditions (Meyer and Dick, 2010). However, as with all probes there are limitations and caveats in using this class of protein probe. For example for some of them the effect of pH on their sensitivity in different cell compartments can complicate analysis and the dynamic range of their response is generally lower than that of small molecule fluorescent probes (Meyer and Dick, 2010).

An alternative to the use of fluorescent proteins is to improve the selectivity of small molecule fluorescent probes. A good example of this process is the use of the selective reactivity of alkylboronates with hydrogen peroxide (Miller et al., 2007; Dickinson et al, 2011) and their even faster reaction with peroxynitrite (Sikora et al, 2009; Zielonka, et al., 2010) to generate novel fluorescent products. This enables the construction of probes that appear to be selective for these species and that avoid many of the limitations of earlier ROS probes such as DCFH. These probes can also be coupled to functionalities that direct them to particular parts of the cell, such as mitochondria, in order to report on both the nature and location of intracellular production of particular forms of ROS (Dickinson and Chang, 2008; Robinson et al., 2006).

One potential limitation to all optical methods for ROS and redox measurements is that they will not be applicable to most animal models *in vivo* and there is the further problem of the quantification of the levels of a particular ROS. Therefore to complement these approaches, non-optical methods to detect ROS are also required. Among those that can be used now to gain some insight into ROS production *in vivo* is measuring changes in the transcription of

genes that are sensitive to the levels of superoxide or hydrogen peroxide (e.g. Landriscina et al., 2009). While these approaches provide useful information on changes in some ROS *in vivo*, they do not indicate the level of ROS present *in vivo* and a number of other approaches have been used to provide this information. Among these has been the use of spin traps *in vivo* that react selectively with free radicals to generate a relatively stable free radical that can then be detected by electron paramagnetic resonance or by mass spectrometry (Reis et al., 2009; Yue Qian et al., 2005). However this approach can be limited by its low sensitivity and the metabolism of the spin adduct. This general approach is being extended by methods under development that greatly enhance the sensitivity of detection by using the ratiometric analysis of a probe that reacts with particular ROS and its stable reaction product relative to their deuterated internal standards by liquid chromatography and tandem mass spectrometry. The first application of this combined the alkylboronate chemistry discussed above with mitochondrial targeting to quantify the levels of hydrogen peroxide within mitochondria inside living fruit flies (Cochemé et al. in press). Consequently there is cautious optimism that “next generation” specific ROS probes will replace the widely used, but problematic, current generation of reagents and facilitate the measurement of particular ROS selectively *in vivo*.

Conclusions and future perspectives

The difficulty of measuring oxidative damage and the levels of particular ROS *in vivo*, together with the uncertainty of interpretation, mean that much is still unknown about the roles of ROS and oxidative damage in many fundamental biological processes. For example, despite extensive investigation most trials of antioxidants in humans have proven disappointing (Bjelakovic et al., 2008). However, without accompanying biomarker and ROS measurements, most of these studies are inconclusive. We cannot be certain that the lack of efficacy was due to the antioxidants failing to affect the oxidative damage involved in the pathology (Gutteridge and Halliwell, 2010). Alternatively, the compounds may have lowered or abolished oxidative damage but still had no impact on outcome, implying that oxidative damage merely correlates with the pathology and is not causative (Cochemé and Murphy, 2010).

Looking forward, this meeting of researchers in the chemistry and biology of ROS revealed that two significant challenges must be met to help progress our understanding of ROS and oxidative damage in living systems. The first challenge is to transmit to a wider audience the knowledge we have already assembled so that it can be used to formulate more precise and testable hypotheses about the role of specific ROS and antioxidants in the laboratory and clinic. The second challenge is to develop better approaches for detecting and quantifying different types of ROS and markers of a range of forms of oxidative damage in biological systems, particularly *in vivo*. There was cautious optimism amongst the group that a more rigorous approach would enable faster progress in the field. Although the results may ultimately show that changes in ROS and oxidative damage in some situations are merely epiphenomena, possibly (for example) during aging (Gems and Doonan, 2009), it is also likely that it will reveal unexpected new roles for ROS at the heart of other major biological phenomena both as damaging agents and as important mediators of redox signalling and other cellular processes.

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HIGHLIGHTS

- ROS have dual roles in biology causing damage and acting as signals
- The specific type of ROS involved in a biological process should be made explicit
- Measurement of both oxidative damage and ROS levels is essential
- New approaches to assess ROS and oxidative damage *in vivo* are being developed

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Table 1

Checklist for assessing a role for ROS in biological processes

1. *What is the specific ROS responsible?*
2. *Is the proposed reaction chemically plausible?*
3 .*Is the ROS or antioxidant present at the appropriate location at a sufficient concentration to carry out the proposed reaction?*
4 .*Does altering the amount of the particular ROS or type of oxidative modification thought responsible impact on the pathology or the redox signal?*