SIGNAL TRANSDUCTION BY REACTIVE OXYGEN AND NITROGEN SPECIES: PATHWAYS AND CHEMICAL PRINCIPLES

Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles

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Preface

Henry Jay Forman, Jon Fukuto and Martine Torres

"Research is to see what everybody else has seen and to think what nobody else has thought."

-- Albert Szent-Gyorgyi

Several years ago, one of us put together a book that dealt with various aspects of oxidative stress and introduced the concept of signal transduction by oxidants. Since then, the interest in the mechanisms by which reactive oxygen and nitrogen species (ROS/RNS) can modulate the cell's response has tremendously grown, paralleling the intense efforts towards identifying new signaling pathways in which phosphorylation/dephosphorylation events take center stage. Evidence is now mounting that production of these species by the cells is required for their function from growth to apoptosis and numerous signaling pathways have been identified where the participation of ROS and RNS is apparent (see Chapters 11-14, 16 and 18). Thus, the field is no more limited to the group of free radical aficionados who have pioneered this area of research but has now gone mainstream. While it is satisfactory for those of us who have been working on this topic for a long time, it has the risk of becoming the "fashionable" motto where those molecules, still mysterious to some, become responsible for everything and anything. In a way, it is reminiscent of the discovery of the phorbol ester receptor, that is to say protein kinase C (PKC) in 1977 [1], a major breakthrough in signal transduction that sparked a flurry of papers. Almost everything seemed to be PKC-dependent at that time. Little did we know that PKC come in various flavors, some of which have nothing to do with the initial definition of the enzyme as a lipid and calcium-dependent

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kinase, that there are many other kinases, since then discovered and involved in complex signaling pathways, and that the "specific" inhibitors used in many studies were not that specific. Nevertheless, redox signaling has gained credence and is now on the map to stay. We profess the hope that this book will help researchers avoid some pitfalls by providing the current state of knowledge in the area of redox signaling, including controversies when they exist and future directions and by including information on physiologically relevant chemistry that can be applied across signaling systems. Although the name "reactive species" seems to imply high and non-discriminative reactivity, chemistry principles may help identify where specificity may occur, a particularly critical concept in signaling, which needs to be better understood in redox signaling.

To put things in perspective, we would like to quickly recount the principal findings that led to today's state of research. It is hard to believe that oxygen, this essential element of aerobic life, was not discovered before the late 18th century when Sheele, Priestly and Lavoisier independently isolated gaseous oxygen, initially branded as "fire air". The generally accepted theory of combustion or "Phlogiston theory" continued to exist for some time and was even defended by Priestly for the rest of his life. It took more than a century before the mechanism by which oxygen supports aerobic life was revealed and several competing theories arose early in the last century. Michaelis first proposed that all biological reductions including that of oxygen were univalent [2]. Thus, the production of superoxide anion ($O_2^{\bullet -}$) was indirectly predicted several decades before any demonstration of its existence in biological systems. Nonetheless, "oxygen activation" whereby oxygen is reduced to water in a concerted reaction in which no intermediates are formed, was for a long period of time considered as the major mechanism of oxygen consumption. The concept was in fact validated by the discovery of cytochrome c oxidase, an enzyme that transfers four electrons to oxygen to produce two molecules of water [3] without the release of intermediates. This led to the general assumption that reduced oxygen species were irrelevant in biology. However, the discovery of hydrogenases by Wieland in 1925 refuted Michaelis's theory as these enzymes catalyze the two-electron reduction of oxygen to form hydrogen peroxide (H_2O_2) [4]. This was the first direct evidence for the potential of H_2O_2 production in biology, although Thenard had discovered as early as 1818 that animal tissues could decompose H_2O_2. Perhaps the over century long delay in acceptance of the reality of two-electron reduction of oxygen occurred, in part, because catalase, the mysterious component in Thenard's preparation, was not recognized as a unique enzyme until 1901 [5].

During the 1940's and 1950's, a large number of flavoproteins and metalloproteins were shown to reduce oxygen by two electrons to produce H_2O_2. The discovery that xanthine oxidase, a ubiquitous enzyme, could produce $O_2^{\bullet -}$ provided the first clue that this free radical might be of importance in biology [6]. Nevertheless, the existence of $O_2^{\bullet -}$ was still not fully accepted as many thought that this reaction was a laboratory curiosity due to protein modification during isolation. However, the "nail in the coffin" for $O_2^{\bullet -}$ came in 1969 when McCord and Fridovich discovered an enzyme whose sole purpose was to remove $O_2^{\bullet -}$, i.e. superoxide dismutase (SOD). First isolated from erythrocytes, it was soon after characterized as a ubiquitous enzyme in eukaryotic organisms and their tissues [7]. Subsequently, SOD isoforms were found in all aerobic organisms, giving credence to the idea that $O_2^{\bullet -}$ is generated in biological systems [8]. The search for $O_2^{\bullet -}$ generating systems

showed that, in addition to xanthine oxidase, a number of flavoproteins and metalloproteins could catalyze univalent reduction of oxygen [9-11]. In 1973, Babior and colleagues reported that such a flavoprotein, present in neutrophils, was able to produce $O_2^{\bullet -}$ at the expense of NAPDH in cells "on command", i.e. upon phagocytosis of bacteria, and that this production explained the drastic cyanide-insensitive oxygen consumption associated with phagocytosis [12]. Furthermore, this NADPH oxidase was an essential part of the host defense against bacterial infections as cells from patients with chronic granulomatous disease were deficient in $O_2^{\bullet -}$ production and bacterial killing, the first proof by nature of the *beneficial* role of free radicals in biology. It took several more years to discover that the enzyme was in fact formed of several components, which were in separate compartments in resting cells. Stimulation results in translocation of the cytosolic proteins ($p47^{phox}$/$p67^{phox}$/$p40^{phox}$) to the plasma membrane where they bind to the flavocytochrome ($p22^{phox}$/$gp91^{phox}$) in a stable complex, competent for electron transfer (see Chapter 6). Unfortunately, soluble agents can also activate this enzyme and its products (i.e. $O_2^{\bullet -}$ and H_2O_2) are not released then within the confine of the phagolysosomes but in the surrounding tissues resulting in damage, hence the *detrimental* and double edge sword image long associated with $O_2^{\bullet -}$ and free radicals in biology.

In the late 1980s and early 1990s, several lines of evidence coming from three distinct fields merged and led to the demonstration of the endogenous generation of nitric oxide (NO) by endothelial cells and the finding that NO, a small diatomic free radical could activate guanylate cyclase, resulting in a dramatic rise in cGMP in the adjacent smooth muscle tissue and ensuing vasorelaxation [13-15] . This established NO as a critical regulator of vascular tone and as a signaling intermediate, the first demonstration of such role for a reactive species [16]. The importance of these findings was affirmed by the awarding of the Nobel Prize in Physiology or Medicine to Drs. Lou Ignarro, Ferid Murad and Robert Furchgott in 1998 for their discovery. The role of NO in physiology is not limited to smooth muscle relaxation as activated macrophages, epithelial cells, and other cell types can also produce NO. Biosynthesis of NO occurs via enzymatic oxidation of the amino acid L-arginine [17-19]. Several nitric oxide synthases (NOS) were identified that had some tissue specificity and particular properties such as the inducible character of the enzyme found in immune cells (iNOS) (see Chapter 7). In these cells, NO appears to participate in the elimination of various infectious agents, as demonstrated in the iNOS knockout model. However, the exact mechanism by which NO exerts its effect in the immune system and in functions other than vasodilation in other cells remains uncertain, as cGMP production does not account for all its effects. This remains one of the most intriguing and active topics in nitrogen oxide biology. NO is also produced by neuronal cells via an analogous biosynthetic pathway [20]. However, as in the immune response, the exact mechanism through which NO functions in the nervous system is not well defined. Understanding the complex chemistry of NO may help elucidate these mechanisms (see Chapter 4).

The finding that a free radical could participate in the production of a second messenger and in the activation of a signaling pathway was of paramount importance as it opened up minds to the idea that the role of reactive species may be more extensive in normal physiology. This idea had previously been put forth about a quarter century ago when exogenous H_2O_2 was shown to mimic the action of

insulin growth factor [21] and, a few years later, when Mukherjeee and coworkers showed that insulin and nerve growth factor stimulated endogenous H_2O_2 production [22]. At that time, the research emphasis was on delineating the involvement of ROS in various pathologies and the identification in biological systems of novel redox agents, resulting from the interaction between ROS and RNS, dictated by their particular chemistry. Another drawback for the expansion of the redox field was the lack of clear understanding of how and where endogenous ROS were produced. Generation of H_2O_2 by mitochondria was discovered in the 1960's [23,24] and in the mid 1970's, several groups demonstrated that H_2O_2 generation by the mitochondrial electron transport chain occurred through the obligatory univalent reduction of oxygen to $O_2^{\cdot-}$ [25-27]. Thus, a so-called leak from the mitochondrial chain is frequently cited as being responsible for increased cellular ROS. However, the production of $O_2^{\cdot-}$ (the generally assumed reaction being via semiquinone autooxidation) has an equilibrium constant that does not favor $O_2^{\cdot-}$ production, and is thereby thermodynamically unfavorable[13]. This means that the $O_2^{\cdot-}$ production is not spontaneous, as often stated, but can only occur if the equilibrium is shifted by coupling to a second reaction such as the dismutation of $O_2^{\cdot-}$ to H_2O_2. Thus, $O_2^{\cdot-}$ is a very transient intermediate, and H_2O_2 production by mitochondria is catalyzed by mitochondrial SOD [16];

$$QH^{\cdot} + O_2 \rightleftharpoons Q + H^+ + O_2^{\cdot-}$$

$$2H^+ + 2O_2^{\cdot-} \xrightarrow{SOD} H_2O_2 + O_2$$

Interestingly, this is one of the few situations in biology in which SOD has been demonstrated to cause an increase in H_2O_2 generation [17]. The production of H_2O_2 by mitochondria has been shown to be dependent upon oxygen concentration; however, an increase in H2O2 can only be observed in a range of O_2 concentrations well above normal physiology [28,29]. In what would appear to be a contradiction of the known dependence on oxygen concentration, increased production of ROS by mitochondria was suggested to be involved in hypoxic signaling [30,31]. Thus, it was not entirely surprising to see that an alternative explanation appeared soon after that did not involve ROS. These recent studies have indicated that activation of the HIFα transcription factor, which modulates most hypoxic adaptation, is accomplished through enzymatic hydroxylation of proline and asparagine that signals for degradation and interaction with the transcriptional apparatus, respectively [32-35]. Thus, as society learned after the 1960's, radicals are not everywhere!

Nonetheless, there is more to mitochondrial H_2O_2 production than its dependence upon oxygen concentration and the role of NO as a regulator of mitochondrial activity and the discovery of a mitochondrial NOS (see Chapters 15 and 17) has provided further insights. Low levels of NO were shown to bind tightly to cytochrome c oxidase, which may increase oxygen at the mitochondrial inner membrane and possibly increase $O_2^{\cdot-}$ production through reduction of Complex III[36] (see Chapter 15). Nevertheless, if NO is present in higher amounts, it may react with $O_2^{\cdot-}$, a reaction that is faster than enzyme-catalyzed $O_2^{\cdot-}$ dismutation [16], and peroxynitrite will be produced (Chapter 4). It has also been suggested that NO and peroxynitrite inhibit Complex III in a similar manner to antimycin A and thereby

promote $O_2^{\cdot-}$ and H_2O_2 production [36,37] (see Chapter 17). Nevertheless, the question remains as to whether mitochondrial H_2O_2 production is regulated in a manner consistent with a role in signaling and further studies will be needed to understand the relationship between NO and regulated production of ROS by mitochondria. In the mean time, the discovery that homologs of $gp91^{phox}$ or NOX proteins are expressed in many cell types and that agents such as angiotensin can induce the regulated production of $O_2^{\cdot-}$ in non-phagocytic cells has given further credence to the role of ROS in signaling (Chapter 6)..

The book previously edited by one of us was entitled "Oxidative Stress and Signal Transduction". The title for this new edition was changed to reflect our perception of redox signaling as events that occur when low levels of ROS are produced and when the targeting of signaling intermediates by reactive oxygen and nitrogen species is specific, transient and required for information to flow through a specific signaling pathway. The involvement of ROS in the EGF and PDGF signaling pathways seems to imply such definition. In contrast, we view signaling during oxidative stress as a response to cell injury, possibly with limited specificity as to the type of stress. Oxidation of a protein cysteine to a sulfinic acid (SO_2H) or a sulfonic acid (SO_3H), or oxidation of bases in DNA are modifications that involve oxidation but are either irreparable or require multiple enzymatic steps for repair that do not involve redox chemistry. Such damage to cellular constituents can stimulate signaling pathways leading to repair or even adaptation; however, these pathways may also be stimulated by damage that is independent of oxidation. Thus, the difference between oxidative stress signaling and redox signaling is not defined by whether cells die because physiological signaling may lead to cell death, albeit regulated as during development (described in Chapters 12, 19 and 20) but rather by the specificity of the response being due to redox chemistry rather than a recognition of damaged cellular constituents. Nonetheless, the boundary between redox chemistry and oxidative stress signaling is sometimes blurred as when a lipid peroxidation product, such as 4-hydroxy-2,3-nonenal, acts as a second messenger (see Chapter 10). The next big challenge for the field of redox signaling will be the identification of the chemical alterations imposed upon signaling proteins by ROS/RNS (or products derived from their action) and how such modification can affect the biological activity of the target, whether it is a kinase, a phosphatase or others. In addition, showing specificity will also be critical. One site of action of ROS/RNS that has long been recognized is the heme iron of enzymes, such as in the interaction of H_2O_2 with catalase and of NO with guanylate cyclase. The interaction of NO with cytochrome oxidase (see Chapter 15) or of H_2O_2 and RNS with cyclooxygenase (see Chapter 13) have also been described to affect signaling. Not surprisingly, thiol chemistry also plays a major role (See Chapters 1-3, 5 and 9). As H_2O_2 does not significantly react with protonated thiols, the oxidation of thiols by H_2O_2 most likely involves thiolates ($-S^-$) to produce a sulfenic acid (-SOH):

$$Protein-S^- + H_2O_2 \rightarrow Protein-SOH + OH^-$$

This requirement for a thiolate, only present in particular electrostatic fields, and the partial oxidation may provide specificity and reversibility that both characterize

a signaling pathway. Glutathione can conjugate to protein thiols through two mechanisms resulting in formation of protein mixed disulfides:

$$GSSG + Protein - S^- \rightleftharpoons Protein - SSG + GS^-$$
$$GSH + Protein - SO^- \rightarrow Protein - SSG + OH^-$$

Thiols can also react with NO to form S-nitroso (S-NO) adducts. This reaction is often called "S-nitrosylation." Others refer to that process as "S-nitrosation," but this implies addition of a nitrosium ion NO^+ when the mechanism of formation of S-NO is still uncertain (Chapter 8). It could be argued then that S-nitrosylation implies addition of NO^- and that a more proper terminology would be "S-nitroso-ylation," which does not imply any particular mechanism but just the addition of an NO residue. As "S-nitrosylation" seems to have gained acceptance as the descriptive term for the formation of S-NO, this should be the common terminology. In fact, the posttranslational modifications regulating the activity of signaling proteins are usually described by the suffix, "ylation", as in phosphorylation, farnesylation, or ribosylation. Thus, we propose that the formation of a sulfenic acid be called "S-hydroxylation," and that "glutathionylation" be used to refer to the formation of mixed disulfides for consistency with other posttranslational modifications involved in signaling.

The last few years have seen exciting development in the area of signal transduction and redox signaling. We anticipate that the coming years will see the "consecration" of reactive species as signaling entities and that further studies will help better understand how dysregulation of ROS/RNS production may alter physiological pathways and lead to disease states. We are grateful to all the authors of this book for their generous contribution and salute their past and future efforts for advancing research in redox signaling.

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