Reactive oxygen species as cellular messengers

Reactive oxygen species (ROSs) have recently been found to be important signaling molecules in several cellular responses. Individual species have characteristic reactive properties, yet are easily interconverted, making it difficult to identify the ROSs involved in each response.

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Reactive oxygen species (ROSs) are presently thought to be important in an increasing number of physiological and pathological processes. They are most often cast in the negative role of damaging cell components, but they also have positive effects in the defense against microorganisms. Recently, a new area of ROS biology has become apparent, with the recognition that ROSs can be intracellular messengers.

The term ROS loosely groups oxygen molecules in different states of oxido-reduction and electronic excitation, as well as compounds of oxygen with hydrogen, chlorine and nitrogen (with the exception of NO, which is now in a class by itself). These species have very different properties and reactivities. To add to the complexity, all ROSs interconvert freely, making it even harder to assign the cause of an experimental observation to the correct guilty party. It is also worth noting that an ROS that is toxic in high doses may be beneficial or indispensable at low concentration, and that to have an effect, an ROS must have both a high reactivity towards a specific target and a chance to reach it. This often requires a compromise between reactivity and diffusion distance.

This review will first briefly trace, in the simplest terms, the chemical lineage of the most important ROSs. We will then discuss how the chemical properties of ROSs correlate with biological function and how the participation of a particular ROS in a biological system can be established. In the second part, we will focus in more detail on three exciting areas in which ROSs have dramatic effects on eukaryotic cells at levels far below those required for cytotoxicity: SAR (systemic acquired resistance in plants), the activation mechanism of the transcription factor NF-κB, and programmed cell death (apoptosis). In these fast moving fields, we cannot provide a comprehensive review; instead, we hope to attract attention to new and intriguing roles for oxygen species.

The cast

From a chemical point of view, it is remarkable that the chemistry of all aerobic life should be based on a molecule as unreactive as oxygen, O_2. In the molecular orbital interpretation of oxygen, two of its twelve electrons are unpaired, and occupy spaces in the highest unfilled orbital of the molecule at opposite ends of the molecule, creating in effect two free radical centers. Thus oxygen is a diradical, in keeping with its paramagnetism. The two unpaired electrons have parallel spins in the most stable configuration of the molecule, which is therefore (by definition) in a triplet state, $^3O_2$. It reacts easily with other paramagnetic ions or molecules; examples are the binding of oxygen to the ferrous ion of hemoglobin or the reaction of $^3O_2$ with free radicals in lipid peroxidation. Such reactions are spin-allowed. But the rules of spin conservation create a barrier to reaction of triplet species with singlet, ordinary diamagnetic molecules, in which all the electrons are paired. Thus molecular oxygen is, relatively speaking, inert.

There are two ways that $^3O_2$ can become reactive, either by acquiring an electron to become O_2^- or by flipping the spin of one of the two unpaired electrons to become singlet oxygen, $^1O_2$. Either of these opens up the enormous array of reactive possibilities diagrammed in Figure 1. If $^3O_2$ becomes $^1O_2$ (reaction *1* in Fig. 1), the two formerly unpaired electrons are now paired, and consequently stay close to each other [1,2]. Conversion of $^3O_2$ to $^1O_2$ requires the expense of 22.7 kcal mol^{-1}, corresponding to a near infrared photon (1270 nm) or to three times the energy released in the hydrolysis of ATP to ADP. This energy can come from a variety of chemical or photochemical reactions, *in vivo* as well as *in vitro*, one of the most common being energy transfer to oxygen from another molecule (a sensitizer, Sens*, in Fig. 1), itself previously excited by light.

Compared to other molecules in electronically excited singlet states, $^1O_2$ has an exceptionally long lifetime, ~2 μsec in water. Because the transition to the triplet ground state is spin-forbidden, an isolated $^1O_2$ molecule protected against deactivating collisions lasts 45 minutes before losing its excitation energy by emitting a 1270 nm photon, analogous to the phosphorescence of an organic molecule, which requires the prototypical spin-orbit emission (from a triplet state to a singlet ground state). In contrast to $^3O_2$, $^1O_2$ reacts fast and selectively with high electron density sites in many unsaturated organic compounds via spin-allowed processes resulting in various peroxides (hydroperoxides and endoperoxides) [1,3]; these can react further, often yielding free radicals.

When $^3O_2$, the ultimate electron acceptor in enzymatic systems, acquires an extra electron in an exothermic

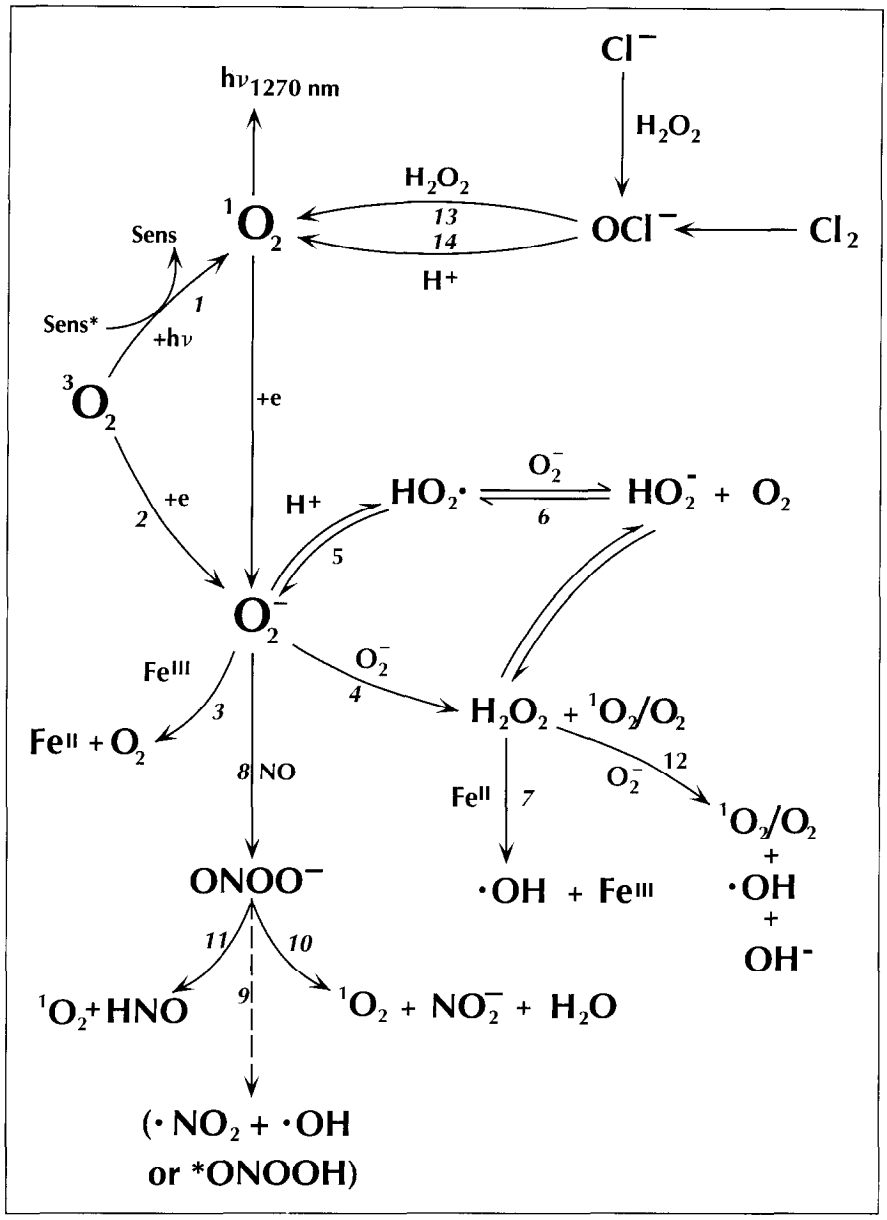

Fig. 1. The main reactions creating, consuming and interconverting ROSs. The numbered processes are further described in the text. Sens*, sensitizer.

process (reaction *2*), for example from semiquinones (intermediates in the reduction of ubiquinone), or from flavins or in the xanthine/xanthine oxidase system, the resulting superoxide anion, O_2^-, is a free radical and thus also a paramagnetic species. Although its name suggests that it is highly reactive, superoxide is unreactive towards most organic molecules and is not an oxidant at neutral pH. It is highly solvated in water and thus is not expected to be transported through membranes or to penetrate into hydrophobic regions of cells and peroxidize lipids. It is, however, a moderately efficient one-electron reductant, for example of transition-metal complexes. This is the basis of analytical methods used to identify superoxide, such as the cytochrome *c* method (reaction *3*), which involves the reduction of Fe^{III} to Fe^{II}. Formation of Fe^{II} via reaction *3* used to be regarded as the critical step in pathways responsible for the toxicity of O_2 (see reaction 7 below), but reductants such as glutathione are more likely to reduce Fe^{III} within the cell.

Unlike two oxygen molecules, two O_2^- molecules undergo dismutation in water to produce two species: one is either $^3O_2$ or $^1O_2$, the other a doubly reduced species, hydrogen peroxide (reaction *4*) [4]. The rate of this spontaneous dismutation is maximal at pH 4.8 ($k = 8 \times 10^7\ M^{-1}s^{-1}$) and is still fast, although two orders of magnitude slower, at physiological pH. Superoxide dismutases (SOD) catalyze this reaction in the the wide pH range of 5.3–9.5, with $k = 1.6 \times 10^9\ M^{-1}s^{-1}$, and thus keep the concentration of superoxide extremely low [5].

O_2^- is a weak base. Its protonation (reaction *5*) gives the hydroperoxy radical (HO_2•), which becomes the dominant species at low pH ($pK_a = 4.8$). Although it is a better oxidant than superoxide, HO_2• is still not a very reactive radical. Superoxide and HO_2• disproportionate (reaction *6*) to give the basic form of hydrogen peroxide, HO_2^-. At neutral or acidic pH, hydrogen peroxide exists almost entirely in the fully protonated form, H_2O_2. In contrast to O_2^- and HO_2•, hydrogen peroxide is a good

oxidant, a well established germicide and is cytotoxic via a variety of mechanisms. It is the substrate of heme peroxidases, which generate a host of free radicals and excited state molecules [6], and of catalase, which reduces it extremely quickly to H_2O and O_2. Catalase is most abundant in the peroxisomes, where H_2O_2 is generated by a variety of oxidases which catalyze the two-electron reduction of oxygen.

Hydrogen peroxide qualifies well as an ROS and a potential signaling molecule. It is a small and stable molecule, which carries no charge; this allows it to cross membranes and to travel freely to its target in cells. Yet it readily participates in one-electron processes. The classic example is the Fenton reaction (reaction *7* in Fig. 1) with metal ions (conveniently reduced by superoxide, reaction *3*), which generates hydroxyl radical •OH, probably via several intermediates. •OH reacts with practically every biological molecule, by abstracting hydrogen or adding to double bonds, often initiating chain reactions.

This brings up an old question: if neither O_2^- nor its protonated form are particularly reactive species, why has nature found it necessary to design the ubiquitous and indispensable superoxide dismutases? The question is even more interesting because the uncatalyzed dismutation of superoxide is already fast under physiological conditions, as noted above, and so the basal level of O_2^- should be low. Moreover, the product of dismutation, H_2O_2, is far from innocuous. Indeed, it has been suggested that in some situations SOD and catalase may act in concert to modulate the cellular concentration of H_2O_2 [7]. The prevalent explanation for the need to reduce O_2^- concentrations in the cell is that O_2^- gives rise to other more reactive ROSs, such as the hydroxyl radical. But •OH reacts with most organic compounds so indiscriminately and fast that its radius of diffusion in cells is expected to be only a few angstroms. Although it might do considerable local damage, •OH is ill-equipped to be a selective, target-specific ROS.

Clearly, other candidates for the critical ROS derived from O_2^- need to be considered. Two deserve particular attention. One is the peroxynitrite anion, $ONOO^-$, formed at diffusion-limited rate in the reaction of superoxide with nitric oxide (reaction *8*) [8]. The other is singlet oxygen.

Peroxynitrite might well be the toxic ROS responsible for some of the effects that neither NO nor O_2^- alone can produce [9,10]. Activated macrophages, for example, generate high concentrations of both •NO and O_2^-, and therefore presumably produce peroxynitrite, which may well be responsible for some of their cytotoxic function. Peroxynitrite is fairly stable under physiological conditions, has a long lifetime and diffusion radius, is a strong oxidant of lipids and many other biological molecules and may take part in the early oxidative modification of low density lipoprotein (LDL) leading to atherosclerotic lesions. Its very weak O–O bond (only half as strong as the already weak O–O bond in hydrogen peroxide [11]) breaks up easily (reaction *9* in Fig. 1) to give $•NO_2$ and an intermediate with •OH reactivity [12,13] (it has also been suggested that ONOOH rearranges to an uncharacterized 'activated form'). Spectroscopic results establish that $^1O_2$ is generated in the reaction of peroxynitrite with H_2O_2 (reaction *10*) [14] as well as, quantitatively, in the acidification of alkaline solutions of peroxynitrite (reaction *11*) [15].

By eliminating superoxide, SOD thus suppresses the formation of $ONOO^-$ and reduces the production of $^1O_2$ from it and from reaction *4*. Activated neutrophils and macrophages produce significant amounts of singlet oxygen [16,17] and several other reactions of superoxide, such as one of the steps of the Haber–Weiss cycle (reaction *12*), have been proven by spectroscopy to be sources of $^1O_2$ [18]. It is hard to evaluate how significant this process is in physiological conditions, especially since both water and superoxide are excellent quenchers of $^1O_2$ and reaction *12* is slow. Furthermore, the chemical effects of singlet oxygen and of hydroxyl radicals will be amplified downstream by secondary free radical reactions. $^1O_2$ reacts with unsaturated compounds to form labile peroxides and hydroperoxides, and •OH abstracts hydrogen atoms from many substrates and initiates autoxidation chains. Thus, even if only a small amount of $^1O_2$ survives quenching, it may still have a significant effect.

A list of biologically active ROSs would be incomplete without the hypochlorite ion, OCl^-, which is generated by such enzymes as myeloperoxidase from Cl^- and the cosubstrate H_2O_2. OCl^- is a strong oxidant that directly peroxidizes and/or chlorinates many biological molecules. Reaction *13* (Fig. 1) also produces $^1O_2$, which then participates in the bacteriocidal system of neutrophils in which myeloperoxidase is the most abundant protein. Even simple acidification of OCl^- generates singlet oxygen (reaction *14*) [19].

Who does what, and how can one tell?

As shown in Figure 1, the ROSs interconvert easily and in an interconnecting pattern. It is therefore no easy matter to determine who does what. Because O_2^- can generate H_2O_2, $^1O_2$, •OH, peroxynitrite and hypohalite ions, it is clearly an important species in spite of the fact that it has very limited reactivity and is unable to penetrate into hydrophobic cell environments. In contrast, •OH is too reactive to reach specific targets, although any reaction of •OH may generate other free radicals that can initiate autoxidation. Hydrogen peroxide is thought to have important functions *in vivo* (see below). Singlet oxygen, the ROS least talked about in the biological literature, must have important cellular functions. It is generated in many ubiquitous processes in cells and it has a particularly favorable balance of a high but selective reactivity with a long lifetime and high diffusion coefficient.

How can one identify the ROS responsible for a given effect? Only spectroscopic methods, such as electron

paramagnetic resonance (EPR) in the case of O_2^- and emission at 1270 nm in the case of $^1O_2$, can provide incontrovertible evidence. But identification is not indictment, and an ROS should be considered innocent until proven guilty.

The next most reliable method for identifying specific ROSs are chemical traps, which result in the formation of reaction products that are characteristic of one specific ROS. One such test has been applied to the detection and quantitation of singlet oxygen generated by neutrophils and macrophages [16,17]. It is based on the thermally reversible reaction of $^1O_2$ (but not O_2^-, •OH or H_2O_2) with 9,10-diphenylanthracene to form a transannular peroxide, shifting its absorption spectrum. Similarly, polynoidin, a protein extracted from the bioluminescent system of scale worms, is a sensitive probe for superoxide, as it emits light *in vitro* when triggered by O_2^- [20].

Quenchers (or antioxidants) are extremely useful for detecting the presence of ROSs; *N*-acetyl-L-cysteine (NAC), for example, is widely used *in vivo*. But caution is needed in interpreting these data. For example, the discovery of SOD focused attention on the functions of superoxide. As discussed above, SOD's effects are often due not to the suppression of O_2^- *per se*, but rather the suppression of nastier ROSs generated by superoxide; moreover, SOD also quenches $^1O_2$ at a diffusion controlled rate, and so some of SOD's effects may be due to the prevention of the damage done by $^1O_2$ [21]. Furthermore, if an ROS is extremely reactive, no reasonable concentration of a quencher, however specific, will be able to prevent it from attacking normal cellular constituents. Solubility can also cause complications in the interpretation of quenching experiments. For example, β-carotene quenches singlet oxygen at a diffusion-limited rate, but requires a hydrophobic environment, whereas vitamin C and SOD need an aqueous medium, and probucol and vitamin E (α-tocopherol) favor the interface between a polar and a non-polar environment.

Recent work [22] illustrates the pitfalls of antioxidant studies. The radical-initiated oxidation of human low-density lipoprotein (LDL) can actually be facilitated instead of retarded by vitamin E, depending on the state of dispersion of the lipid in aqueous solution. Oxidation of LDL occurs by a free-radical chain mechanism carried on by peroxy radicals, and these radicals are efficiently scavenged by vitamin E. When vitamin E intercepts a peroxy radical, a new, relatively inert, free radical is formed. This radical can abstract a hydrogen atom from a fatty acid moiety of LDL and initiate a new chain, unless it is eliminated by reacting with vitamin C (or with another radical, in a chain-terminating step).

Because of these pitfalls the evidence from antioxidant (quencher) studies can generally only be considered to be circumstantial. Nevertheless, if such evidence incriminates a particular ROS, the case may be strengthened if addition of this ROS enhances the effects. Some examples of studies in which the ROS involved has been at least tentatively identified are discussed below.

ROSs as second messengers: plant systemic acquired resistance

Plants have an elaborate two-stage mechanism of defense against pathogens. Following infection, an immediate, brief and localized oxidative burst occurs, which probably directly kills some of the attacking pathogens. In a matter of minutes, the build up of H_2O_2 at the surface of the cell causes cross-linking of cell wall proteins and forms a physical barrier, impeding pathogen entry [23]. Some of the time, the cell initially attacked undergoes programmed cell death, triggered by high internal levels of either H_2O_2 or trapped pathogens [24]. This localized oxidative burst is known as the 'hypersensitive response'. A second line of defense, in which a number of pathogenesis-resistance (PR) genes are activated in the initially attacked cell, follows this early response by hours or days. The PR genes are then activated in neighboring cells, and eventually throughout the plant [25]. These genes are responsible for the appearance of a generalized protection of the whole plant against the original pathogen as well as against other agents; this protection, termed 'systemic acquired resistance' (SAR), lasts for weeks or months [26] . Since different pathogens activate the same plant resistance genes, they must act via a common pathway in which H_2O_2 or other ROS appear to have a role (see Fig. 2).

Salicylic acid was proposed as a candidate for the signal transducer on the basis of several convergent lines of evidence [27–29]. Attack by a pathogen such as the tobacco mosaic virus increases the level of endogenous salicylic acid throughout the plant (although these increases are slight; the data are difficult to interpret because salicylic acid is stored as an inactive glucoside and also compartmentalized in the chloroplasts [30]). The time scale of this increase is consistent with that of SAR. Exogenous salicylic acid induces the PR genes and activates SAR. Moreover, tobacco plants engineered to express salicylic acid hydroxylase, and therefore unable to accumulate salicylic acid, fail to develop SAR [31]; in contrast, in an *Arabidopsis* mutant with an elevated endogenous level of salicylic acid, SAR is constitutive [32]. A soluble protein that binds salicylic acid, SABP, was isolated, which not only shares a high sequence identity with some catalases, but acts *in vitro* as a catalase. Binding of salicylic acid to SABP inhibits its catalase activity [33]. The induction of SAR by salicylic acid or its analogs, such as aspirin, correlates with the ability to bind to SABP and inactivate its catalase activity. Consistent with this, the concentration of H_2O_2 was shown to increase in leaves treated with salicylic acid.

Taken together, these observations indicate that salicylic acid is required for the activation of the resistance genes. It does not seem to be the mobile signal that initiates PR gene expression in the rest of the plant, however. This was shown in an experiment in which wild-type

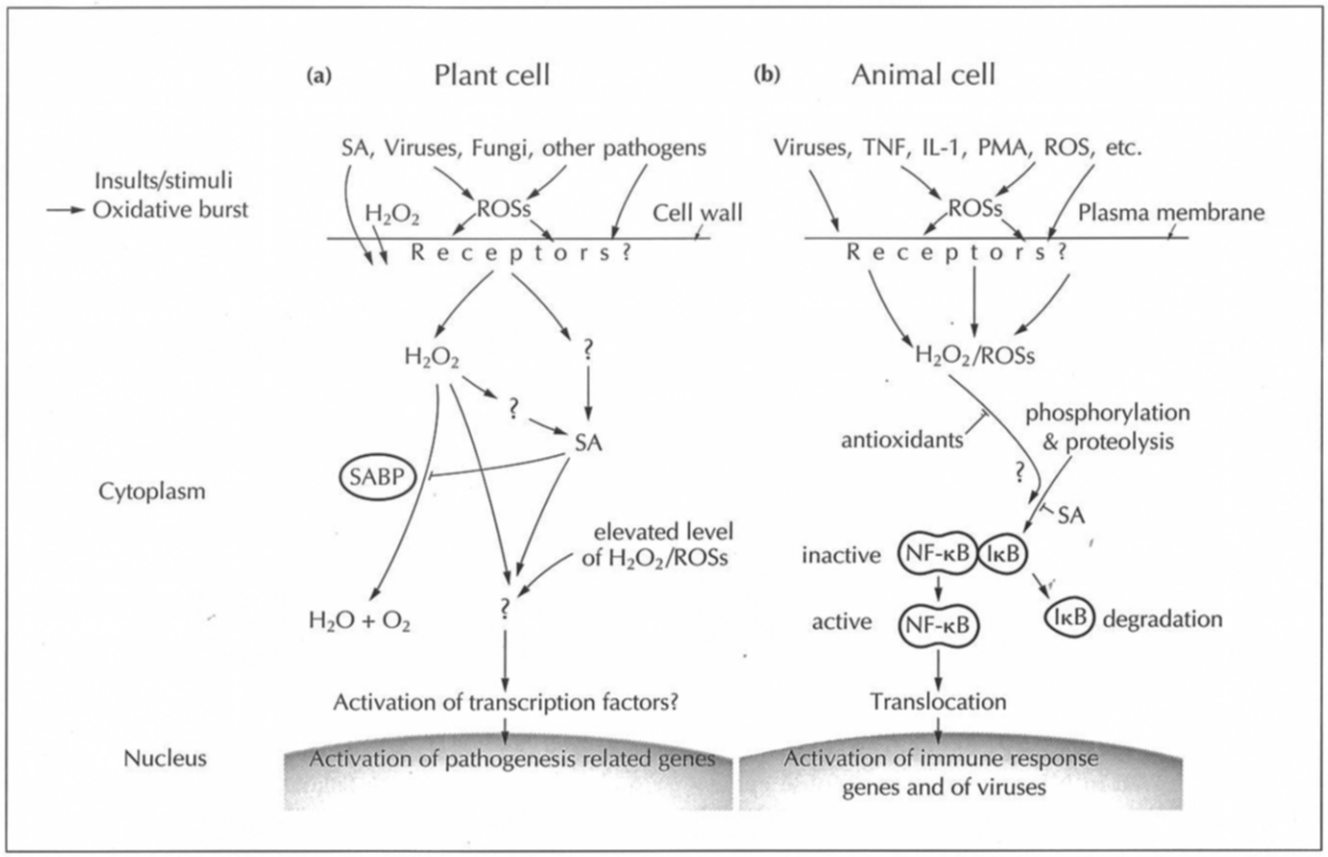

Fig. 2. ROSs as second messengers. **(a)** Plant systemic acquired response (SAR). The first response to a variety of insults is an immediate oxidative burst which generates ROSs and results in cross-linking of cell wall proteins. A diffusible signal, as yet unidentified, subsequently spreads through the cell and the whole plant, and the cytoplasmic level of the signal molecule salicylic acid (SA) rises. SA binds to the catalase SABP, inhibiting its activity and increasing the intracellular concentration of H_2O_2. The cause of activation of the pathogenesis-related genes is still unclear. **(b)** In animal cells (fibroblasts, lymphoma cells, etc.), the common response to a variety of stimuli is also an elevation of the H_2O_2 level. Possibly as a result, the inhibitory protein IκB, which forms a complex with and inactivates the transcription factor NF-κB, is phosphorylated, then proteolytically degraded. This allows NF-κB to enter the nucleus, bind to the immune response genes and activate their transcription. Salicylic acid (SA) and aspirin inhibit the dissociation of IκB and thus gene activation. Some viruses, such as HIV-1, use the NF-κB–IκB system to modulate their own transcription.

tobacco plants were grafted onto plants that expressed salicylic acid hydroxylase, and were thus unable to develop SAR [30]. The stocks, which were unable to accumulate salicylic acid, were treated with tobacco mosaic virus. Although the stocks did not develop SAR, the wild-type grafts did. Thus, an as yet unidentified diffusible signal responds to the initial oxidative burst even in the salicylic acid depleted tissue, and travels through the graft junctions to induce the salicylic acid mediated SAR in distal tissues. This signal molecule may itself be an ROS. In any case, the increased level of H_2O_2 caused by the down-regulation of the catalase activity of SABP upon salicylic acid binding could trigger the expression of defense-related genes. Thus hydrogen peroxide, or another ROS derived from it, may act as second messengers; recent results with antioxidants are inconclusive [34], and the role of H_2O_2 in SAR is still debated [35].

One question that arises from this work is whether the plant genes are activated directly by ROSs. Another possibility is that the gene activation might be mediated by an ROS-activated inducible transcription factor, similar to the NF-κB–IκB complex in animal cells [36], which will be discussed below.

ROSs and the activation of NF-κB

NF-κB is a protein complex that activates the transcription of protective genes involved in inflammation and infection in response to a variety of stimuli (Fig. 2) [37]. These include exposure to UV, heat shock, viruses, H_2O_2, cytokines such as IL-1 and the tumor necrosis factor (TNF), phorbol esters (PMA), and inhibitors of protein kinases and phosphatases, such as okadaic acid. Some of these stimuli act by binding to specific cell-surface receptors. The signals from these various stimuli appear to converge on a common pathway, along which the inactive multiprotein complex NF-κB–IκB is converted to its active form by the release of the inhibitory protein IκB [38]. The active NF-κB complex is translocated into the nucleus where it binds to DNA and induces gene expression (Fig. 2). Although the precise mechanism controlling the degradation and/or release of IκB is not fully elucidated, there is strong evidence pointing to phosphorylation of IκB [39,40] as the step

that regulates its proteolytic degradation [41]. Some viruses, such as HIV-1, take advantage of the NF-κB–IκB system to induce their own transcription.

Many, if not all, of the disparate stimuli listed above have the common effect of producing ROSs in the cytoplasm. This has been convincingly established for TNF and IL-1, which have been shown to cause the release of O_2^- in fibroblasts [42], as well as for phorbol esters, which activate protein kinase C leading to an increase in the production of O_2^- by NADPH oxidase. It has been suggested that ROSs, which may be present extracellularly in inflammation, may in all cases act as second messengers and activate NF-κB. Three lines of evidence support this hypothesis [43]. First, in some cell lines, H_2O_2 induces expression of the genes controlled by NF-κB (and of no other genes) as strongly as does TNF or PMA. Second, direct treatment of T-cells with H_2O_2 activates the binding of NF-κB to DNA (both of these effects may be enhanced by the metabolism of H_2O_2 into a more reactive ROS *in vivo*). Third, quenchers of ROSs such as NAC and pyrrolidine dithiocarbamate inhibit the activation of NF-κB *in vitro*.

A recent experiment provides a clue to the identity of the ROS involved in NF-κB activation. Mouse epidermal cells overexpressing either catalase or SOD were compared to controls in their response to TNF or okadaic acid. Catalase decreased the activation of NF-κB, whereas SOD increased it; since SOD generates H_2O_2, the opposite effects of overexpressing these two enzymes point to H_2O_2 as the possible activating agent [7]. It is not known whether IκB is selectively damaged by the relevant ROS or, more likely, whether the ROS activates a redox-sensitive kinase (tyrosine kinase has been suggested [39]) that targets IκB for protease degradation. But the conclusion seems clear that ROSs can act as second messengers between various stimuli and NF-κB. This conclusion should not come as a surprise, since some ROSs (such as H_2O_2 and singlet oxygen) share with nitric oxide many of the attributes desirable in a second messenger: small size, absence of charge, ubiquity in cells, a limited lifetime and precise regulation via both synthesis and degradation enzymes.

In view of the central role of salicylic acid and ROSs in the activation of plant defense genes, it is intriguing that salicylic acid and aspirin have recently been shown to modulate the activation of NF-κB by preventing the degradation of IκB [44]. The suggestion of a new role for aspirin-like drugs in reducing inflammation (other than by inhibiting prostaglandin synthesis) is provocative. In plants, the binding of salicylic acid to SABP increases the concentration of H_2O_2, inducing ROS-mediated gene expression; in mammalian cells, in contrast, salicylic acid prevents gene transcription by inhibiting the release of IκB via a still unknown process, and therefore the entry of NF-κB in the nucleus. In both systems, H_2O_2 seems to transduce the intracellular signal.

ROSs and apoptosis

Although there is no consensus on the possible role of ROSs in apoptosis, and conflicting results appear weekly, the data reported to date warrant discussion. The gene-encoded program of orderly cell death is a key feature of embryonic development and control of cell number in multicellular eukaryotes. For example, in the nematode *Caenorhabditis elegans*, exactly 131 of the initial complement of 1090 cells undergo apoptosis during development. Generally, the morphological characteristics of this death by suicide include chromatin condensation, DNA degradation into nucleosomal fragments, nuclear collapse, whole cell shrinkage and surface blebbing, with no concomitant architectural alteration of the organelles. The cell eventually fragments into apoptotic bodies, which are phagocytosed by neighboring or specialized cells. If for a variety of pathological reasons apoptosis is inhibited, the undue proliferation of cells leads to tumors and cancers, whereas increased apoptosis contributes to AIDS and to a host of neurodegenerative diseases. One school of thought regards apoptosis as the default intracellular program, survival depending on input from other cells [45–47].

Apoptosis can be induced by a wide spectrum of insults, such as radiation, growth factor removal, glucocorticoids and elevation of Ca^{2+} via ionophores, all acting along convergent routes of activation. Remarkably, in all these cases, mammalian cell death can be prevented by the expression of the proto-oncogene *bcl-2*. [48]. The protein it expresses must therefore act downstream from the convergence point of the activation pathways, but upstream from the morphological changes associated with apoptosis. The *bcl-2* gene is similar in sequence, structure and function to the *ced-9* gene of *C. elegans*, which also prevents cell death [49]. This, together with the fact that a protein from baculovirus, p35, prevents death of mammalian as well as insect cells, suggests that the genetic program of apoptosis may have been largely conserved throughout evolution [46].

Bcl-2 localizes to the endoplasmic reticulum and to mitochondria, probably to the outer membrane of the latter or at junctional complexes where outer and inner membranes abut. Since mitochondria are major sites of ROS generation, the possibility that Bcl-2 might act as an antioxidant was investigated. It does [50–52]. For example, in model systems (such as neural cells or thymocytes) the antioxidant NAC prevents apoptosis in activated cells. After treatments that lower the intracellular concentration of reduced glutathione, producing a relatively oxidizing environment in the cell, cells expressing Bcl-2 survive better than those lacking Bcl-2; in contrast, overexpression of Mn-SOD affords no protection [51]. Bcl-2 protects cells from H_2O_2 (up to 0.5 mM) and from menadione-induced oxidative deaths, but Bcl-2 has no peroxidase activity *in vitro* [50] and does not act on pathways linked to oxidative phosphorylation. Following treatment with the apoptosis activator glucocorticoid dexamethasone, Bcl-2 was

found to prevent lipid peroxidation and, in parallel, DNA fragmentation.

A provocative report suggests that Bcl-2 may function neither by scavenging ROSs nor by decreasing their production, but paradoxically by acting as a pro-oxidant [53]. Bcl-2 was expressed in an *Escherichia coli* mutant strain lacking SOD, and its resistance to apoptotic death induced by a brief treatment with H_2O_2 was compared with that of a strain in which Bcl-2 is not expressed. Bcl-2 was found to increase survival by a factor of 20. At the same time, the final concentration of H_2O_2 was 80 times lower in the Bcl-2 strain than in the control, due to an increase in the concentration of the inducible catalase KatG in the periplasm of the Bcl-2 strain. The induction of *katG* is attributed to an oxidative stress present throughout aerobic growth only in the strain expressing Bcl-2; indeed, KatG is indispensable to the growth and survival of these cells.

Bcl-2 can therefore be regarded as indirectly regulating the level of H_2O_2. It accomplishes this in a counter-intuitive way, not by acting as an antioxidant, but by creating oxidative stress and increasing the concentration of ROSs, which in turn induces the catalase gene. If the concentration of ROSs required to induce *katG* expression is small, Bcl-2 has thus used a sub-toxic concentration of H_2O_2 as a messenger to cause the induction of high levels of catalase, keeping the level of ROSs below that causing apoptosis.

Experiments with T-cells from a line of acute lymphoblastic leukemia argue persuasively for a role for H_2O_2 as a second messenger [54]. Low density cultures of these cells in serum-free medium undergo fast apoptosis, unlike high density cultures or cultures supplemented with conditioned medium, in which the cells proliferate. The factor in the conditioned medium allowing cell growth was identified as human catalase; catalases from other sources also afforded protection. The catalase activity released in the medium is minute compared to the amount of catalases always present intracellularly, mostly in the peroxisomes. Yet, it is this trace amount of extracellular catalase which protects them against extracellular H_2O_2. T-cell survival *in vivo* must indeed depend on protection from ROSs generated by neighboring macrophages and neutrophils.

On the basis of experiments at low oxygen tension, two recent reports concluded that ROSs are not involved in apoptosis [55,56]. However, the argument is not as strong as it might seem, since it is well known that reactions of molecular oxygen can still proceed efficiently *in vivo* at even lower concentrations of O_2, 10 nM in the case of the bioluminescence of some bacteria [57]. Since apoptosis evolved early and appears to be conserved, the apoptotic program might well have been already in place when oxygen levels were low [46].

Spermine, and to a lesser extent spermidine, were also shown to inhibit endonuclease-activated fragmentation of DNA and apoptosis induced by Ca^{2+} ionophores and glucocorticoids in thymocytes, and it was suggested that these polyamines act by preserving the integrity of the the chromatin structure [58]. These polyamines are also known to be singlet oxygen quenchers, however, and can protect DNA from $^1O_2$-induced single-strand breaks [59,60]. Thus, spermine and spermidine may inhibit apoptosis indirectly, by lowering the cellular level of an ROS.

What next?

Given the network of processes linking oxygen reactive species with one another, attempts at intercepting a single ROS are not likely to be fruitful from the viewpoint of understanding biochemical mechanisms or developing therapeutic strategies. Yet once the involvement of ROSs is securely established, a deeper understanding can only come from precise identification of which ROS is responsible. In the three examples discussed here, it seems clear that ROSs do indeed act as messengers. It is striking that in all three systems the suspect is hydrogen peroxide. Of all the ROSs shown in Figure 1, only H_2O_2 and $^1O_2$ are possible candidates, since the others are either too unselectively reactive, or so solvated that they cannot be expected to go through membranes, or both.

How does hydrogen peroxide, or a derived ROS, activate the SAR genes and NF-κB? It is worth remembering that NO, the best-understood signaling molecule of this type, has many similarities with small uncharged ROSs, and that the pathways forming these species are interlinked (Fig. 1). Once the active species is known, the search for the enzymes that it activates or inhibits via oxidative processes will have a chance of succeeding, as it did in the case of NO. The redox chemistry of NO is now understood to have a major part in the modulation of the activity of NO as a signaling molecule [61]. The development of new NMR methods based on ^{17}O may help to unambiguously identify individual ROSs.

We have chosen to focus here on the newly discovered role of ROSs as messengers. The roles of ROSs in cytotoxicity and cell-to-cell communication are equally important and fascinating, but are also more often reviewed. For an overview of these subjects, see [62].

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