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Beyond oxidative stress: an immunologist's guide to reactive oxygen species

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Abstract

Reactive oxygen species (ROS) react preferentially with certain atoms to modulate functions ranging from cell homeostasis to cell death. Molecular actions include both inhibition and activation of proteins, mutagenesis of DNA and activation of gene transcription. Cellular actions include promotion or suppression of inflammation, immunity and carcinogenesis. ROS help the host to compete against microorganisms and are also involved in intermicrobial competition. ROS chemistry and their pleiotropy make them difficult to localize, to quantify and to manipulate — challenges we must overcome to translate ROS biology into medical advances.

The term 'reactive oxygen species' (ROS) includes super-oxide, hydrogen peroxide, singlet oxygen, ozone, hypo-halous acids and organic peroxides[1]. ROS participate in phenomena that traverse all of biology, and their study has burgeoned for more than a century (TIMELINE). ROS are difficult to distinguish from each other by specific assays and are challenging to quantify. The diversity of their enzymatic sources has only recently become apparent, and tools for the identification of their subcellular localization are only now emerging. Many of their effects can be opposed to one another — for example, they can both promote and prevent cell death, inflammation or ageing.

Further complicating their study, ROS are not the only class of endogenous small, reactive signalling molecules; other classes include reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$) and $NO_2^{\bullet}$; hydrogen sulphide (H_2S) or its anion HS^-; and carbon monoxide (CO). These other reactive molecules can have properties that both overlap with and are

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The authors declare no competing financial interests.

Note added in proof
Two recent reports[154,155] demonstrated that killing of *Escherichia coli* by norfloxacin, ofloxacin, kanamycin or ampicillin does not involve ROS.

FURTHER INFORMATION
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distinct from those of ROS. Scientists who study ROS and RNS organize separate conferences, but the molecules themselves interact and affect the production and targets of one another[3–5].

Most medical interventions that target ROS have failed (discussed in REF. 6). This can be interpreted to mean that ROS have an unimportant role in pathophysiology; however, it might also be that the interventions tested were not based on an adequate understanding of ROS biochemistry and biology, which is still emerging[7]. Moreover, among immunologists who consider specificity the hallmark of the discipline, some labour under the misconception that ROS are nonspecific, and a few cling to the view that only phagocytes produce ROS and that the only function of ROS is to kill pathogens and host cells.

Thus, it is no surprise that until recently it was rare to find a diagram of signal transduction or a systems biology analysis that took ROS into account. However, in the past few years, many of the obstacles mentioned above have been overcome. Scientists are now aware that ROS routinely arise in most cells from defined sources, that they affect multiple targets in specific ways and that they exert considerable influence over cell function.

In this Review, we describe ROS in terms of their regulation, targets and actions. Because the topic is vast, our approach is illustrative rather than comprehensive. Given the conservation of ROS biology, we draw lessons from diverse fields to lend perspective to the role of ROS in immunology.

ROS homeostasis

Many authors state that ‘oxidative stress’ occurs when the production of ROS exceeds their catabolism. However, the term stress is an imprecise reference to a restricted range of ROS signalling that runs from adaptive to maladaptive (FIG. 1) and has a major role in cell and organismal biology[1,8–10] (FIG. 1). Thus, oxidative stress is by no means a description of, nor a synonym for, the biology of ROS.

Sources of ROS

Endogenous sources of ROS in mammals (BOX 1) include seven isoforms of NADPH oxidases (NOXs)[10,11] that are differentially expressed in diverse cells and species[12]; the mitochondrial respiratory chain; the flavoenzyme ERO1 in the endoplasmic reticulum; xanthine oxidase; lipoxygenases; cyclooxygenases; cyto-chrome P450s; a flavin-dependent demethylase; oxidases for polyamines and amino acids; and nitric oxide synthases. Free copper ions or iron ions that are released from iron–sulphur clusters, haem groups or metal storage proteins can convert $O_2^{\bullet -}$ and/or H_2O_2 to $OH^{\bullet}$ (REF. 13).

Box 1

Sources of ROS and mediators of their catabolism

Exogenous sources of ROS

- Smoke
- Air pollutants

- Ultraviolet radiation
- γ-irradiation
- Several drugs

Endogenous sources of ROS

- NADPH oxidases
- Mitochondria
- ER flavoenzyme ERO1
- Xanthine oxidase
- Lipoxygenases
- Cyclooxygenases
- Cytochrome P450 enzymes
- Flavin-dependent demethylase
- Polyamine and amino acid oxidases
- Nitric oxide synthases
- Free iron or copper ions
- Haem groups
- Metal storage proteins

Catabolism by antioxidant systems

- Superoxide dismutases
- Catalases
- Glutathione peroxidases
- Glutathione reductase
- Thioredoxins
- Thioredoxin reductases
- Methionine sulphoxide reductases
- Peroxiredoxins or peroxynitrite reductases

Catabolism by small molecules that react with ROS non-enzymatically

- Ascorbate
- Pyruvate
- α-ketoglutarate
- Oxaloacetate

ER, endoplasmic reticulum; ROS, reactive oxygen species. Sources reviewed in REFS 10–12.

Regulation of ROS production

Several checkpoints restrict ROS production by the NOXs to times and locations that are appropriate for cellular functions. NOXs are transmembrane flavocytochrome proteins, the cytosolic domains of which transfer an electron from NADPH to a FAD cofactor. From there, the electron is passed to a haem group, which donates it to O_2 on the extracellular side of the membrane, generating $O_2^{\bullet -}$. One control step in this process is the loading of the apoprotein with the flavin cofactor[14]. In another level of regulation, Ca^{2+} signalling, phosphorylation cascades and the activation of small G proteins control the recruitment of accessory proteins from the cytosol to join the flavocytochrome at the membrane, forming the functional NOX complex[8]. NOXs are activated following the activation of receptors by ligands such as insulin, platelet-derived growth factor, nerve growth factor, fibroblast growth factor, chemokines that bind G protein-coupled receptors, tumour necrosis factor (TNF), granulocyte–macrophage colony-stimulating factor (GM-CSF), angiotensin, sphingosine-1-phosphate, lysophospholipids, complement component 5a (C5a) and leukotriene B4 (LTB4), as well as by cell adhesion and by phagocytosis[1,8]. The mechanisms linking receptor interaction to NOX activation are an important research area in immune signalling.

ROS production by mitochondria is regulated by diverse factors, including RNS, mammalian target of rapamycin (mTOR), p53, SHC-transforming protein 1 (SHC1), reactive oxygen species modulator 1 (ROMO1), B cell lymphoma 2 (BCL-2) family members[8] and uncoupling proteins[15]. Another factor that promotes mitochondrial ROS generation is hypoxia (discussed below).

Nitric oxide synthases (NOS) can produce ROS when concentrations of their cofactor tetrahydrobiopterin or their cosubstrate L-arginine are limiting. For example, following L-arginine depletion by L-arginase, NOS donate some of their NADPH-derived electrons to O_2 rather than passing all the electrons to the guanidino nitrogens of arginine, generating both $O_2^{\bullet -}$ and $NO^{\bullet}$. These species react with each other to produce peroxynitrite ($OONO^-$), which decomposes to generate the strong oxidants OH^- and NO_2^- (REF. 16), and can oxidize redox-sensitive cysteines 10^3-fold faster than peroxide[17].

Transcriptional networks control proteins that sense and regulate the uptake and the storage of redox-active metal ions[13]. In addition, other mechanisms that affect the intracellular levels of ROS include the export of xenobiotics that generate ROS[13] and the export of ROS themselves into neighbouring cells through connexin channels[18].

Catabolism of ROS

Until recently, the antioxidant systems of a cell were thought to be superoxide dismutases, catalases and the enzymes of the glutathione redox cycle, which couples the reduction of peroxide to the oxidation and the regeneration of reduced glutathione. Many additional physiologically important ROS-regulating enzymes are now recognized, among them

thioredoxins, thioredoxin reductases[19], peroxiredoxins (which also function as peroxynitrite reductases) and methionine sulphoxide reductases[8,20,21]. Moreover, recent studies have increased our understanding of glutathione homeostasis. For example, the level of reduced glutathione is preserved in yeast not only through the action of glutathione reductase, but also through the actions of thioredoxin reductase and glutaredoxin, and the export of oxidized glutathione into the vacuole[22].

Pyruvate kinase, an enzyme involved in glycolysis and gluconeogenesis, is crucial in the negative feedback regulation of ROS. When cells metabolize glucose, ROS inhibit pyruvate kinase by oxidizing Cys358. The resulting inhibition of glycolysis directs carbon flux into the pentose phosphate pathway. This increases the production of NADPH and sustains the reduction of oxidized glutathione and thioredoxin, returning ROS to homeostatic levels[23].

In addition, many small molecules that react with ROS non-enzymatically can be recycled or replenished, giving them a ROS-buffering capacity. These include ascorbate and the α-ketoacids of central carbon metabolism, such as pyruvate, α-ketoglutarate and oxaloacetate[24] (BOX 1). Just as some molecules that are better known for other functions can also function as antioxidants, so molecules that are mainly known as antioxidants can have other functions. For example, when released from cells, thioredoxin is a potent chemoattractant[25] and peroxiredoxins can trigger inflammation[26].

Repair of ROS-mediated damage

Cells maintain homeostasis despite ROS production not only by catabolizing ROS but also by repairing oxidative injury. For example, ROS can oxidize the sulphurs that hold the iron atoms in the iron–sulphur clusters. As a result, iron atoms are lost. The apoprotein then loses its original function and might have to be re-synthesized for a new iron–sulphur cluster to be attached. DNA oxidation by ROS activates the nucleotide excision repair and base excision repair pathways[13].

Some oxidative damage cannot be repaired, such as the formation of carbonyl groups on amino acid side chains in proteins. This results in cells degrading irreversibly oxidized macromolecules using the proteasome[27] or autophagosomes[28]. Some macromolecules might be so extensively oxidized that they can be neither repaired nor degraded; cells might respond by sequestering these macromolecules with chaperones. Thus, it is not surprising that a genetic screen of viable deletion strains of *Saccharomyces cerevisiae* for hypersensitivity to ROS identified 456 genes[29]. Many of these genes encoded proteins with additional functions to catabolism of ROS, repair of ROS-dependent damage and the other mechanisms discussed above.

The fact that regulators of ROS are encoded by such a large proportion of the genome and are distributed across so many functional classes reflects the widespread functional effects of ROS. It seems unlikely that evolution selected for fitness by ascribing such a widespread role to molecules that react nonspecifically. We argue below that the biological effects of ROS are in fact highly specific.

Targets of ROS

Atomic targets

ROS display a type of specificity that is atomic rather than molecular: ROS react covalently, often reversibly, with only certain atomic elements in macromolecules, and with only a subset of those atoms[30] (FIG. 2). Therefore, ROS only seem to react nonspecifically if we limit our ideas of specificity in signalling to molecular 'handshakes' that depend on complementarity, as in the case of insulin binding to its receptor. Such handshakes are well suited to the transmission of a signal along a discrete pathway. By contrast, reactions of ROS with specific atoms that are present in many macromolecules might transmit signals to multiple pathways at once. Depending on the origin and level of ROS, this might occur in discrete subcellular locations or across a large proportion of the cell. ROS that are produced by a local source in small enough amounts to be confined to a restricted subcellular location can function as a rheostat for discrete signalling pathways in that location. ROS that are produced in large enough amounts to diffuse across more of the cell can function as coordinators of global signalling in the cell. After ROS have accumulated to a certain level, their activating effects might give way to inhibitory effects. As a result, the diverse signalling pathways that are simultaneously regulated by cellular ROS levels are associated with the metabolic state of the cell[30].

Consistent with the idea of 'atomic specificity', one of the atoms with which ROS most often reversibly reacts in cell signalling — sulphur — is one of the least abundant atoms in biological macromolecules. Even then, ROS do not react with all sulphur atoms, but mostly with a subset of sulphur atoms in the side chains of cysteine or methionine residues in peptides or proteins. Many of the most ROS-reactive cysteines in proteins are located in environments that are conducive to the participation of the thiolate in active site chemistry. The reactivity of specific cysteinyl thiols is partly influenced by neighbouring amino acid side chains that confer an acidic dissociation constant (low pKa), but additional factors are also involved that remain to be identified[31]. Much remains to be learned about this important form of intracellular signalling[32].

Molecular targets: proteins

A partial list of proteins that have been reported to be physiologically regulated by ROS includes tyrosine and serine/threonine phosphatases, such as phosphatase and tensin homologue (PTEN)[33]; tyrosine and serine/threonine kinases, such as epidermal growth factor receptor[34], protein kinase B[35], ataxia-telangiectasia mutated (ATM) kinase[36], calmodulin-dependent kinase II[37] and protein kinase G-Iα[38]; zinc-finger proteins[39]; other transcription factors, including those of the forkhead box O (FOXO) family[40]; histone deacetylases[41]; signal-regulating binding proteins, such as heat-shock proteins[41]; caspases[41]; metalloproteinases[42]; protease inhibitors, such as α1-antitrypsin[43], α2-macroglobulin[44] and secretory leukocyte protease inhibitor[45]; metabolic enzymes; prolyl hydroxylase[46] and glucose uptake regulator[47], which both depend on α-ketoglutarate as a cofactor; GTP cyclohydrolase[48]; guanylyl cyclase[49]; and ion channels, such as the ryanodine receptor[50].

An important role of ROS in signal transduction is to transiently oxidize the cysteine sulphydryl that contributes to the active site of most phosphatases. The phosphorylation that follows the binding of a ligand to its receptor is usually attributed to the activation of a kinase, but can also be the result of a burst of ROS formation and the transient inactivation of cognate phosphatases. Proteome-wide assessment has shown that ROS regulation of phosphatases, and hence of phosphorylation, is widespread[51].

In other proteins, ROS sensing by cysteine residues can provide feedback control to regulate the levels of ROS. For example, the intermolecular oxidation of conserved cysteines in the transcription factor FOXO4 allows for the binding of this protein to the p300/CBP acetylase, which leads to lysine acetylation of FOXO4. Such a phenomenon might be related to the ROS-dependent transcriptional induction of manganese superoxide dismutase, catalase, peroxiredoxin and sulphiredoxin, and to the reduction in levels of ROS[40,52]. Similarly, oxidation of important cysteine residues in ATM kinase promotes glucose flux through the pentose phosphate shunt, increasing the levels of NADPH. NADPH is the physiological reductant for oxidized glutathione and thioredoxin, and thus, the ultimate reductant for many ROS-catabolizing enzymes, such as glutathione reductase, peroxiredoxins and methionine sulphoxide reductases[53]. Regulation of ROS seems to be important for the functions of ATM in haematopoiesis[54] and neoangiogenesis[55].

A main function of ROS is their contribution to the activation of transcription by several mechanisms. For example, the bacterial transcription factor SoxR is activated by the superoxide anion, which interacts with the iron–sulphur redox centre of SoxR, whereas another transcription factor, OxyR, is activated by H_2O_2, which oxidizes the cysteinyl thiol of OxyR. Both events lead to the expression of antioxidant enzymes[56]. Moreover, ROS-facilitated protein phosphorylation can lead to the kinase-mediated activation of a transcription factor such as JUN, or to the translocation to the nucleus of a cytosolic transcription factor such as nuclear factor-κB (NF-κB) following the kinase-triggered ubiquitylation and subsequent degradation of its inhibitor (FIG. 3).

ROS can also promote transcription by increasing the accumulation of transcription factors via inhibition of their degradation. For example, increased production of ROS by hypoxic mitochondria (FIG. 4) has an important role in preventing the degradation of hypoxia-inducible factor 1α (HIF1α), which is the main transcription factor that cells use to adapt to the hypoxic state[57–59]. In turn, HIF1a mediates hypoxic transition to glycolytic metabolism, reducing electron flow through mitochondria and reducing the associated production of ROS. Thus, the generation of reactive oxygen intermediates (ROIs; a subset of ROS) under hypoxic conditions should not be viewed as a wasteful process carried out by a poorly evolved system, but rather as an elegant network, through which the reduction in the availability of a crucial molecule triggers transcriptional adaptation.

Molecular targets: DNA

Mitochondrial ROS can promote transcription by oxidizing DNA itself, rather than by oxidizing DNA-binding regulatory proteins (FIG. 5a). For example, ROS that are produced by hypoxic mitochondria oxidize specific bases in the promoter of vascular endothelial growth factor (*VEGF*). This enhances binding of HIF1α to the *VEGF* promoter[60]. Evidence

for the physiological relevance of this response comes from the observation in pulmonary artery endothelial cells that hypoxia triggers dynein-dependent movement of mitochondria along microtubules so that the mitochondria cluster around the nucleus. As a result, mitochondrial-dependent ROS promote oxidative modifications of, and HIF1α binding to, the *VEGF* promoter[61]. Thus, ROS are physiological mediators in signalling between mitochondria and the nucleus[9].

In another example of gene regulation through DNA oxidation (FIG. 5b), transcriptional activation by the oestrogen receptor, the androgen receptor, the retinoic acid receptor, the thyroxin receptor and activator protein 1 all require topoisomerase IIb-mediated, site-specific DNA breaks in target gene-regulatory regions[62]. ROS are involved in the formation of these breaks. The oestrogen receptor-activated flavin-dependent lysine-specific histone demethylase 1 (LSD1; also known as KDM1A) produces H_2O_2 (REF. 63). This locally produced H_2O_2 oxidizes bases in target gene-regulatory regions, forming 8-oxoguanine. 8-oxoguanine recruits 8-oxoguanine DNA glycosylase 1 (OGG1), which causes single-stranded DNA breaks. These breaks recruit topoisomerase IIb, which bends DNA while repairing the lesion, thereby enabling the transcription initiation complex to access the targeted promoters[63]. The same process seems to be required for MYC-mediated transcription[64].

Thus, ROS arising from NOXs at the plasma, endosomal and phagolysosomal membranes, and from mitochondria in the cytosol, can influence transcription by regulating the phosphorylation of transcription factors; whereas ROS arising from perinuclear mitochondria or from a nuclear flavoenzyme can participate in transcriptional control by targeting DNA directly. This recent understanding of ROS–DNA interactions complements the long-standing recognition of the mutagenic potential of ROS.

Effects of ROS in the immune system

In the immune system, ROS are neither unique products of one subset of cells, such as phagocytes, nor do they have one action, such as to kill other cells. Instead, ROS have a physiological role in signalling that probably extends to every cell type in immunology. As with any signalling mechanism, ROS can become cytotoxic if the signal is too strong, if it lasts too long or if it arises at the wrong time or place.

ROS in innate immunity

The first functional role ascribed to the production of ROS by mammalian cells was the killing of microorganisms by phagocytes (TIMELINE). This was confirmed by the discovery that individuals with chronic granulomatous disease (CGD), a disorder characterized by heightened susceptibility to infection, have disease-causing mutations in NOX2, the main source of ROS in polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes.

PMNs migrate to sites of tissue damage in response to chemotactic factors such as interleukin-8 (IL-8), C5a, LTB4, and formyl peptides released by mitochondria or bacteria. All of these can trigger NOX2-dependent H_2O_2 production. It was shown in zebrafish

larvae, where endothelial cells near wounded tissue activate the Nox isoform dual oxidase (Duox), that H_2O_2 itself is a chemotactic factor[65]. One mechanism through which H_2O_2 affects the migration of leukocytes in fish and humans involves the oxidative activation of the tyrosine kinase LYN and perhaps other SRC family kinases[66]. Thus, H_2O_2 might help to direct PMN movement in an autocrine or a paracrine manner.

Moreover, H_2O_2 that is produced in PMNs can contribute to PMN migration by promoting the accumulation of phosphatidylinositol-3,4,5-trisphosphate at the leading edge of the plasma membrane. NOX2 that is localized at the plasma membrane generates H_2O_2, which transiently inactivates the phosphatase PTEN, allowing inositol polyphosphates to accumulate[33]. At the peak of a chemotactic gradient, where migrating PMNs congregate and begin to ingest bacteria, ROS levels are probably much higher and more sustained than near individual PMNs that are beginning to emigrate from the bloodstream. High ROS levels can suppress cell motility by promoting the accumulation of glutathionylated actin, which is impaired for polymerization[67]. Thus, ROS can coordinate the migration of PMNs towards the pathogens that wounding will probably introduce, and then ROS can promote the retention of the PMNs at that site.

Consistent with the potential role of NOX2 in the migration of mammalian PMNs, NOX2-deficient PMNs failed to migrate up a chemotactic gradient *in vitro* or to sites of inflammation *in vivo*[68]. Thus, the immuno-deficiency of CGD not only involves defective bacterial killing, but might also be a result of delayed PMN accumulation. Therefore, it might seem paradoxical that CGD was named for the tendency to form excessive granulomas; however, this might reflect an impaired oxidative inactivation of chemotactic factors, such as C5a, formylated peptides and LTB4 (REFS 69,70). This indicates that ROS might help not only to initiate but also to terminate inflammation.

As mentioned above, host-derived factors, such as IL-8 and C5a, which are induced in response to bacterial products, can trigger ROS production from leukocytes. However, bacteria can also trigger ROS production directly through diverse types of receptors on leukocytes. For example, Toll-like receptor (TLR) engagement can induce ROS production by NOX2 and by other sources that contribute to signalling. NOX2-derived ROS were shown to be required for X-box-binding protein 1 (XBP1)-mediated transcriptional responses downstream of TLR2 and TLR4 that led to control of *Franciscella tularensis*[71]. In macrophages, TLR1, TLR2 and TRL4 engagement recruited mitochondria to phagosomes and promoted mitochondrial H_2O_2 production, which contributed to the control of *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection *in vitro*. Mice expressing mitochondrial catalase had a twofold to threefold greater burden of *S.* Typhimurium 5 days after infection compared with wild-type mice[72]. However, it is not clear whether TLR-induced mitochondrial ROS are required for mice to clear infection.

ROS and inflammasomes

An important question in innate immunity is how structurally diverse agonists activate the same inflammasomes. ROS seem to mediate inflammasome activation in a range of circumstances, although the molecular steps involved and the sources of ROS remain controversial. The late Jurg Tschopp and his colleagues advanced a theory based on the

premise that all known activators of the NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome induce ROS, which lead to the dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin. They proposed that mitochondria are the source of ROS and that the association of TXNIP with NLRP3 is the basis of its activation[73]. Other work indicates that diverse NLRP3-activating stimuli might converge to promote mitochondrial damage. Damaged mitochondria produce excess ROS, which attack the mitochondrial DNA. Oxidized mitochondrial DNA enters the cytosol, and binds to and activates NLRP3 (REF. 74).

Further studies might help to clarify the links between diverse NRLP3-activating stimuli, mitochondrial damage and the resulting ROS overproduction. It is possible that another source of ROS might initiate the mitochondrial damage, in effect beginning a process that then becomes self-sustaining. This might explain why knock down of the common NOX2 subunit p22phox (also known as CY24A) decreased IL-1β secretion in response to a range of inflammasome activators[75]. It remains to be explained what terminates this process that risks initiating a positive feedback loop that could be lethal for the cell. In fact, production of mature IL-1β is closely associated with apoptosis[76].

ROS, the intestinal microbiota and enteric pathogens

In contrast to patients with CGD, mice that are only deficient in NOX2 are not susceptible to spontaneous infections during standard husbandry, which is similar to mice that are only deficient in inducible nitric oxide synthase (NOS2; also known as iNOS). However, mice lacking both NOX2 and NOS2 succumb to spontaneous, invasive infections by their own microbiota in the form of massive abscesses filled with PMNs and monocytes[77]. It seems that the antibiotic proteins and peptides, the acidification pumps, the lysosomal hydrolases and the autophagic mechanisms of PMNs and monocytes are not effective in the infected organs in the absence of both NOX2 and NOS2. This might indicate that both ROS and RNS are required to integrate with the other mechanisms listed above. Indeed, ROS are required for antibacterial autophagy in PMNs, macrophages and some epithelial cells[78]. The partial mutual redundancy of NOX2 and NOS2 obscures the full importance of ROS in controlling infection. Taken together, these data show that NOX2 and NOS2 are essential for mice to coexist with their microbiota[77].

DUOX in colonic epithelial cells also contributes to coexistence of the host with the microbiota. This enzyme produces submicrobicidal levels of ROS in response to commensal bacteria, such as *Lactobacilli* spp., which promote epithelial repair and suppress inflammatory responses[79]. One mechanism of suppression of inflammation by ROS involves the reversible, oxidative inactivation of a component of the neddylation pathway, which prevents the ubiquitylation and the proteasomal degradation of inhibitor-α of NF-κB (IκBα) and the activation of NF-κB[80]. By contrast, high levels of ROS that are produced during intestinal inflammation, along with high levels of RNS, contribute not only to the suppression of microbial growth but also to epithelial injury[79].

Some intestinal pathogens capitalize on inflammatory ROS production. For example, ROS can convert endogenous thiosulphate into tetrathionate, which *S.* Typhimurium can use as an

electron acceptor[81] to achieve a growth advantage in regions of the intestinal lumen that are nearly anoxic[79].

ROS and the regulation of lymphocyte function

ROS were discovered when phagocytosing leukocytes were found to consume large amounts of oxygen by a non-mitochondrial process (TIMELINE). B cells and T cells neither phagocytose nor show large increases in mitochondrial-independent oxygen consumption following activation. Thus, it was assumed that ROS have no role in adaptive immunity, and little notice was paid when superoxide production by the 'phagocyte oxidase' was discovered in transformed[82] and primary[83] human B cells at about one-tenth of the levels of ROS that are released by phagocytes. However, later studies showed that ROS production is functionally important in lymphocytes. Following B cell receptor (BCR) ligation, ROS transiently inactivate BCR-associated phosphatases and function synergistically with calcium transients to potentiate signal transduction[84]. Similarly, T cell receptor (TCR) engagement triggers ROS production, with superoxide and peroxide regulating pro-apoptotic and proliferative pathways, respectively[85]. NOX-derived ROS that are released outside of the plasma membrane or into the lumen of a plasma membrane-derived vesicle enter the T cell via aquaporin 3 to affect cytosolic phosphatases[86]. T cell-derived peroxide also activates NF- B, which leads to the production of IL-2 (REF. 87). T cell activation also indirectly depends on ROS, in that the consumption of protons by NOX2 in the phagosomes of dendritic cells (DCs) retards phagosomal acidification, impedes the action of proteases and preserves peptides of sufficient length to be presented on MHC molecules[88].

Lymphocytes could not be cultured until Mishell and Dutton[89] discovered the trophic effect of supplementing the medium with the reducing agent 2-mercapto-ethanol[89]. Using transformed lymphocytes, Nathan and Terry[90] showed that non-activated macrophages could replace the reducing agent to sustain lymphocyte growth, but that activated, ROS-producing macrophages could not[90] (TIMELINE). Subsequent work established that macrophages and DCs can respond to various stimuli, including contact with antigen-specific T cells, by secreting glutathione, which is broken down to release cysteine, an amino acid that is otherwise scarce in extra-cellular fluid. Cysteine uptake allows the lymphocytes to synthesize their own glutathione, to maintain redox homestasis, and to undergo antigen-specific activation and proliferation[91]. Consistent with the observation that mouse macrophages differ in their secretion of lymphocyte-trophic thiols depending on their activation state[90], lamina propria-resident human macrophages secrete little cysteine, and neighbouring T cells are fairly glutathione-deficient and hyporeactive, whereas the lamina propria is infiltrated by innate immune cells with higher cysteine-releasing capacity during inflammatory bowel disease[92]. Moreover, regulatory T (T_{Reg}) cells can suppress glutathione release by DCs, thereby indirectly regulating the activation potential of other T cells[93].

As noted above, even though ROS help to mediate T cell activation, T cell activation also partly depends on help from accessory cells to maintain T cell levels of the antioxidant glutathione. This indicates that ROS can be immunosuppressive. Indeed, ROS were the first molecules found to suppress T cell function[94]. Some T_{Reg} cells can suppress other T cells through their release of ROS, and T_{Reg} cells can be induced by macrophage-derived

ROS[95,96]. In keeping with these observations, T_{Reg} cells were found to be more resistant to ROS than effector T cells; their enhanced resistance was associated with greater secretion of thioredoxin[97].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that are temporarily restricted from further differentiation. MDSC numbers can increase tenfold in the blood of patients with cancer and inflammatory disorders[98–100]. During cancer progression, the recruitment of MDSCs to the tumour site can suppress immune reactivity[101]. MDSCs produce large quantities of ROS. ROS production not only underlies the immunosuppressive properties of MDSCs but it is also thought to maintain them in an undifferentiated state[99,102]. Nitration of CD8, TCR, and CC-chemokine ligand 2 (CCL2) by MDSC-derived peroxynitrite prevents the binding of $CD8^+$ T cells to peptide-loaded MHC class I, promotes antigen-specific tolerance and impairs cytotoxic T cell recruitment to tumours[101,103]. MDSCs from mice lacking NOX2 demonstrated little or no ROS production and they lacked the ability to suppress T cell proliferation and the production of IFNγ[101,103].

ROS and tumour cells

Once ROS were discovered to be a principal mechanism by which the immune system controls pathogens, investigators asked whether ROS also contribute to the ability of activated macrophages to selectively kill tumour cells. In several mouse models ROS seemed to account for much of the antitumour activity of immunologically activated macrophages[104–106]. Killing could be greatly increased by pharmacologically inhibiting tumour cell glutathione synthesis or glutathione reductase, or by restricting dietary selenium, which is required for the function of glutathione peroxidase[107]. It was even possible to mimic activated macrophages with an artificial, particulate H_2O_2-generating system and to cure mice of an advanced lymphoma[108]. However, human cells proved to be about 100-fold more resistant to ROS than their mouse counterparts[109]. Moreover, cell lines derived from metastatic human tumours were found to produce ROS at high levels[110].

The phenomenon of ROS overproduction by tumour cells has been widely confirmed[111]. In some cases, excessive ROS production has been attributed to mutations in a mitochondrial gene that encodes a component of the mitochondrial electron transport chain. Introduction of those mutations into other tumour cells was sufficient to confer both enhanced ROS generation and ROS-dependent metastatic potential[112]. ROS release by tumour cells might sensitize, or even self-activate, their growth factor receptors by inhibiting the associated tyrosine phosphatases[51,113]. Tumour cell ROS production might also help to explain how tumour cells alter their central carbon metabolism[114] — for example, towards synthesis of nucleic acid precursors[23] — and it might also contribute to immunosuppression. Moreover, the mutagenic actions of ROS that are derived from inflammatory cells can contribute to the initial stages of tumorigenesis[115]. After a tumour is established, ROS derived from radiotherapy, from chemotherapy or from the tumour cells themselves might contribute to the genomic instability of tumour cells[116], fostering drug resistance, just as ROS production that is induced in bacteria by antibiotics can cause mutations that promote antibiotic resistance[117]. Finally, tumour cell-derived ROS can trigger the tumour stroma to produce

angiogenic factors[111]. In contrast to more differentiated cancer cells, cancer stem cells exhibited lower levels of intracellular ROS, greater levels of antioxidant pathways and more efficient DNA repair responses to ionizing radiation[118–120]. Thus, the effects of ROS on tumours can range from tumour-promoting effects to tumour-destroying effects, which means that ROS-producing phagocytes are potentially dangerous cells for both the tumour and the host.

Looking ahead

Two sets of questions loom large over ROS biology: the mechanisms of ROS production and action, and the translational potential of this information in medicine.

Mechanistic mysteries

It remains unclear exactly how cytokines, TLR ligands, inflammasome agonists and lymphocyte antigen receptors trigger ROS production and how the subcellular localization, the magnitude and the duration of ROS production determine their specific functions. Systems biology has yet to integrate ROS biology fully into the ‘wiring’ diagrams that reflect our understanding of cellular behaviour.

These advances will partly depend on the development of tools that would help to identify ROS and their subcellular localization and to quantify them at the level of single cells and single molecular species in real time. These tools are beginning to be developed but the challenges are considerable. Not all redox couples in a cell are maintained at the same equilibrium, and it is not well understood what insulates one couple from another. Some of the older organic dyes that react with ROS to produce or suppress fluorescence lack specificity for individual ROS[121]. Newer approaches that allow for sensitive and specific measurement of ROS include small compounds[122]; novel delivery systems, such as nanotubes[123] and peroxalate micelles[124]; and genetically encoded redox-sensitive fluorescent proteins, such as redox-oxidation-sensitive green fluorescent proteins (roGFPs)[125]. The use of some genetically encoded bio-sensors is limited by their slow or irreversible response to changing redox levels. However, roGFPs that are fused with glutaredoxin can capture real-time changes in the aspect of cellular redox potential that is linked to the redox state of glutathione[126]. Another challenge is to determine the extent of oxidation of specific targets, both per single molecule and as a proportion of the molecules of a given molecular species. Quantitative redox proteomic techniques are struggling with this challenge[121].

Medical advances

The disappointing history of efforts to prevent or to treat disease with exogenous anti-oxidants does not reflect the important role of ROS in pharmacology. Many drugs partly work by generating ROS, by inducing intracellular production of ROS, by sensitizing cells to ROS[107], by diminishing the cellular production of ROS or by increasing the catabolism of ROS[111,127,128].

For example, many antibiotics kill bacteria partly by inducing them to make ROS. β-lactams, which target peptidoglycan synthesis in the bacterial cell wall, and

aminoglycosides, which inhibit the bacterial ribosome, both create metabolic stress that results in NADH auto-oxidation and $O_2^{\bullet -}$ production in bacteria. $O_2^{\bullet -}$ can dislodge Fe^{2+} from iron–sulphur clusters, and Fe^{2+} together with O_2 or H_2O_2 can generate the most potent oxidant known, $OH^{\bullet}$, which contributes to bacterial death[129,130]. In short, a principal biochemical mechanism of host defence that evolved in the immune system over millions of years matches a major form of artificial host defence that was engineered by scientists over the past few decades. This is not a coincidence; it is a consequence of convergent evolution. Most antibiotics in clinical use are, or mimic, natural microbial products — signalling molecules that microorganisms use to communicate with each other. Apparently, similar advantages in fitness against microbial competitors supported the evolution of small, secreted, ROS-inducing molecules in bacteria and large, ROS-generating intra-cellular enzymes in eukaryotes. A better understanding of how antibiotics lead to ROS production as part of their mechanism of action[129] could help the urgent need to revitalize antibiotic research and discovery[131].

Some anti-infectives, such as clofazimine[151], and anti-cancer agents that also have antibiotic actions, such as adriamycin[152] and bleomycin[153], produce ROS directly. In the case of the anticancer agents, ROS production contributes both to their efficacy and to their toxicity.

Among the unanticipated anti-inflammatory actions of statins is their ability to decrease ROS production by endothelial cells. The synthetic oleanoid triterpenoids, one of the newest classes of anti- inflammatory agents, partly work by binding to KEAP1 (Kelch-like ECH-associated protein 1), which releases nuclear-related factor 2 (NRF2; also known as NF2L2) to induce a panoply of endogenous antioxidant defences[132].

Substantial medical advances could result from an increased understanding of how to foster or to inhibit ROS production, and how to monitor the effects of these interventions.

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Glossary

Iron–sulphur clusters	Prosthetic groups that are required for the function of some enzymes. In iron–sulphur clusters two, three or four atoms of iron are attached to the protein through two or four sulphydryl groups
Uncoupling proteins	Proteins in the mitochondrial inner membrane that can divert the proton gradient away from the formation of ATP, resulting in the generation of heat instead
Xenobiotics	Small chemical compounds that enter an organism unnaturally, such as drugs or pollutants

Acidic dissociation constant (pKa)	The equilibrium constant for the dissociation of an acid into its conjugate base and hydrogen ion, expressed as the negative logarithm. The lower the pKa of a sulphydryl group, the greater the likelihood that the sulphur will be anionic at ambient pH
Chronic granulomatous disease (CGD)	An immunodeficiency state manifested by recurrent, often life-threatening, infections and the excessive formation of granulomas, caused by mutations in any one of four subunits of NADP oxidase 2
Granulomas	Histological collections of macrophages, usually surrounded by lymphocytes and sometimes fibrocytes. Some of the macrophages might seem to be ‘epithelioid’ or fuse to become multinucleated giant cells. Granuloma formation is a chronic inflammatory response to various infectious and non-infectious agents
Neddylation	A process that is analogous to ubiquitylation, in which ubiquitin-like protein NEDD8 is conjugated to a protein substrate

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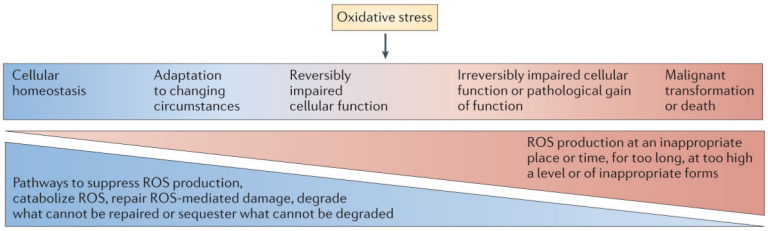


Figure 1. The broad range of ROS signalling is influenced by ROS production and catabolism, and by cellular adaptation

Restriction of reactive oxygen species (ROS) production to appropriate subcellular locations, times, levels, molecular species and for appropriate durations allows ROS to contribute to homeostasis and physiological cell activation. For example, brief pulses of H_2O_2 production at the plasma membrane or at the endosomal membrane mediate signalling in response to the engagement of receptors with cytokines, microbial products or antigens (left-hand side). When ROS production escapes these restrictions — for example, when there are high levels or sustained production of hydroxyl radicals — macromolecules are damaged (‘oxidative stress’). ROS-mediated damage can often be reversed by repair, replacement, degradation or sequestration of the damaged macromolecules (middle). However, damage that exceeds the capacity of the cell for these responses can lead to cell death (right-hand side). When damage to DNA results in mutagenesis without irreparable double-strand breakage, and when damage to other macromolecules is repaired, the consequence can be malignant transformation rather than death of the cell (right-hand side).

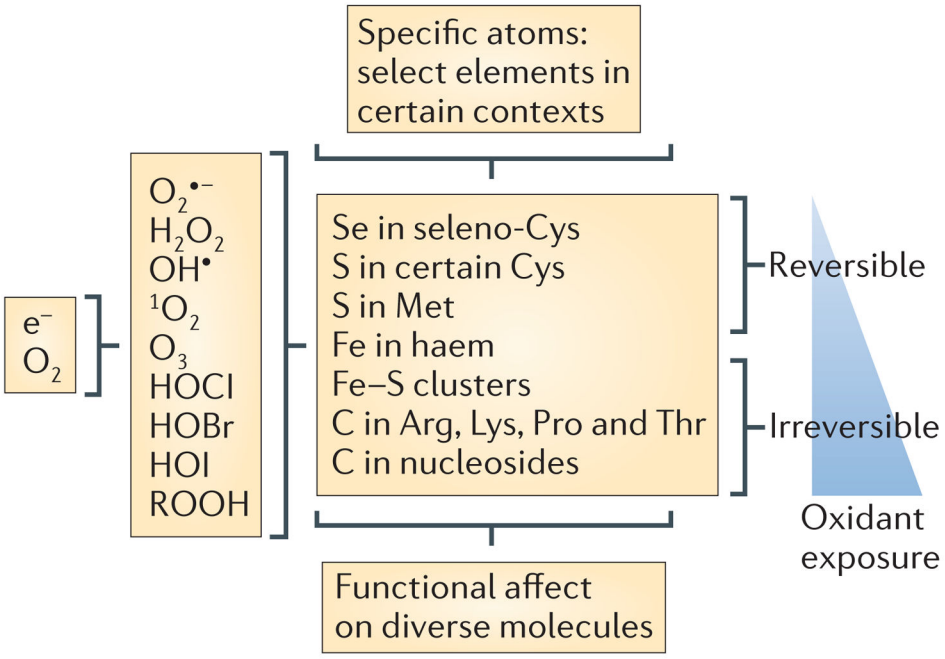


Figure 2. ROS and their atomic specificity

During the reduction of oxygen to water, sequential one-electron reductions can produce reactive oxygen intermediates (ROIs) — superoxide, hydrogen peroxide and hydroxyl radicals — along with singlet oxygen and ozone. ROIs comprise a subset of reactive oxygen species (ROS). Additional ROS are the hypochlorous (HOCl), hypobromous (HOBr) and hypoiodous acids (HOI) that arise when peroxidases catalyse the oxidation of halides by H_2O_2, as well as important products of the reaction of ROS with other molecules that retain strong oxidizing potential, such as lipid peroxides (included as ROOH in the figure). ROS at low levels tend to react reversibly with a limited number of atoms — for example, selenium or sulphur atoms in a subset of cysteine and methionine residues — conferring atomic specificity in reactions involving diverse macromolecules. At higher levels ROS are likely to react irreversibly with certain iron and carbon atoms. e^-, electron.

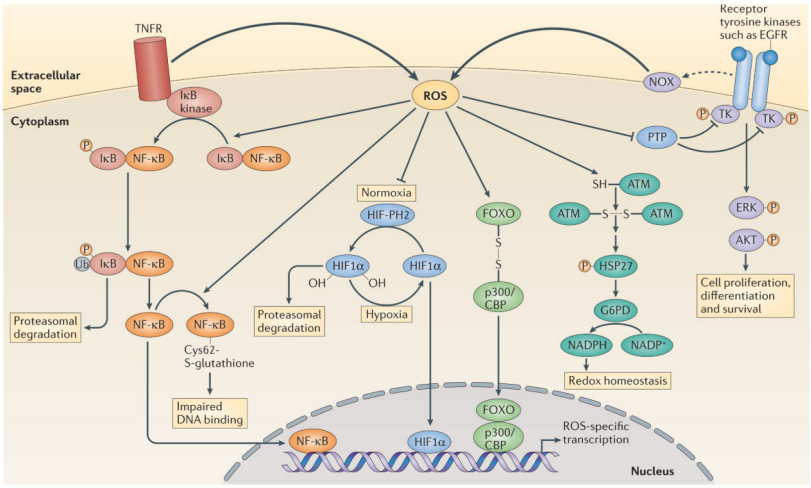


Figure 3. Examples of transcriptional regulation by ROS acting at the plasma membrane or in the cytosol

Activation of tumour necrosis factor receptor (TNFR) triggers reactive oxygen species (ROS) production, which enhances the phosphorylation (P) of inhibitor of NF-κB (IκB), probably through the oxidative inhibition of a phosphatase. This leads to the ubiquitylation (Ub) of IκB and its subsequent degradation by the proteasome. Nuclear factor-κB (NF-κB) is then released and translocates to the nucleus to initiate transcription. ROS production can also trigger oxidative glutathionylation of NF-κB at its redox sensitive cysteine, which reduces its DNA binding affinity[133]. Under normoxia, prolyl hydroxylases (PHs) hydroxylate hypoxia-inducible factor 1α (HIF11α), which allows it to be recognized by the E3 ligase von Hippel–Lindau tumour-suppressor protein (VHL) and promotes its degradation by the proteasome. Under hypoxia there is increased production of ROS (FIG. 4), which inhibits prolyl hydroxylases, leading to the accumulation of HIF11α. HIF11α then translocates to the nucleus to mediate gene transcription. ROS production by NADPH oxidases (NOXs) following receptor activation by specific ligands, for example, epidermal growth factor receptor (EGFR), inhibits protein tyrosine phosphatases (PTPs), which promotes the phosphorylation of tyrosine kinases (TKs) and the subsequent signal transduction. By contrast, ataxia-telangiectasia mutated (ATM) kinase is activated directly by ROS, through disulphide bond-mediated homodimerization, which leads to the phosphorylation of heat shock protein 27 (HSP27) and the subsequent activation of glucose-6-phosphate-dehydrogenase (G6PD). The resulting increase in NADPH levels contributes to the maintenance of cellular redox homeostasis. ROS-mediated disulphide bonding can also lead to heterodimerization, as seen between forkhead box O (FOXO) transcription factors and p300/CBP acetyltransferase, which leads to the acetylation of FOXO proteins and the activation of specific gene transcription. ERK, extracellular signal-regulated kinase.

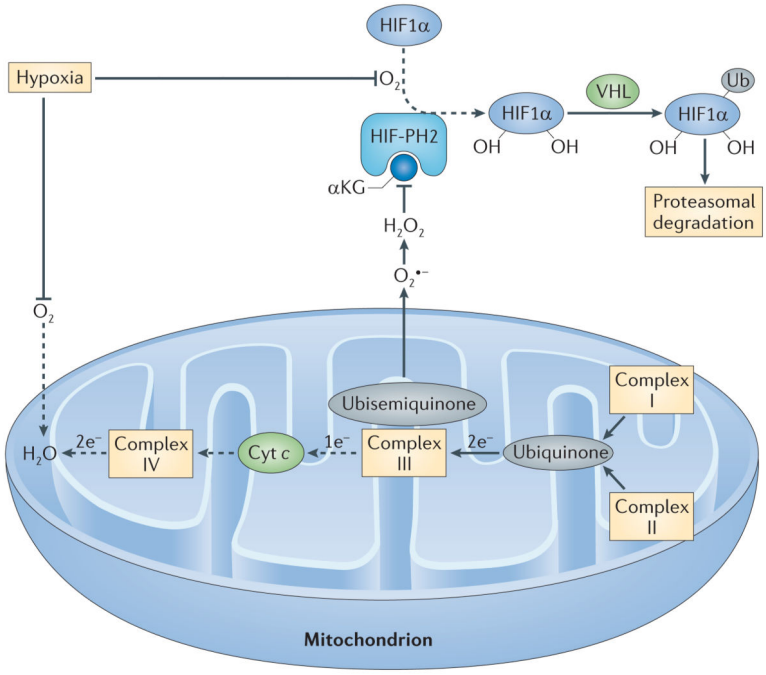


Figure 4. Regulation of HIF1α by mitochondrial ROS production during hypoxia

In the presence of O_2 and its cofactor α-ketoglutarate (αKG), HIF-prolyl hydroxylase 2 (HIF-PH2) hydroxylates two proline residues in hypoxia-inducible factor 1α (HIF1α). Hydroxylated HIF1α is then ubiquitylated (Ub) by the E3 ligase von Hippel–Lindau tumour-suppressor protein (VHL) and is subsequently degraded by the proteasome. Because O_2 is a substrate for HIF-PH2, hypoxia limits HIF-PH2 activity. This limitation is enhanced by the negative effect of hypoxia-driven mitochondrial reactive oxygen species (ROS) on HIF-PH2 function. Complex III in the mitochondrial electron transport chain (METC) receives two electrons from ubiquinone but can only transfer one electron at a time to cytochrome *c* (Cyt *c*). Complex III therefore transfers one electron to a quasi-stable ubisemiquinone radical. If this radical accumulates, O_2 that is dissolved in the mitochondrial membrane can capture the electron before cytochrome *c* accepts it, generating superoxide ($O_2^{\bullet-}$). If cellular O_2 is low, complex IV at the end of the METC is slow to transfer pairs of electrons to O_2 (making water), and the METC is blocked between complex III and IV, which favours the electron leak described above. The newly formed superoxide dismutates to H_2O_2. H_2O_2 can inhibit HIF-PH2 and oxidatively decarboxylate its cofactor, αKG. The resulting decrease in the hydroxylation of HIF1α and its subsequent proteasomal degradation supports HIF1α accumulation. Dashed arrows indicate the reactions that are slowed in hypoxia.

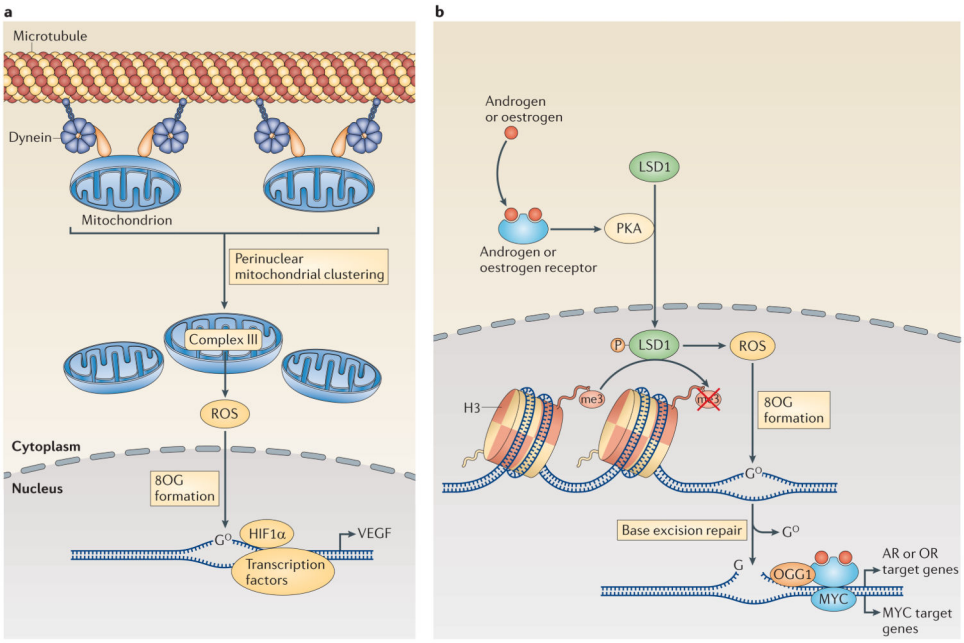
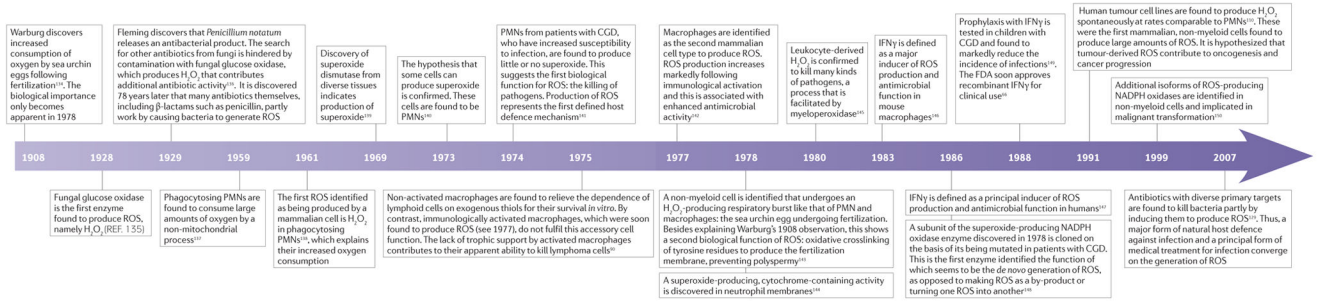


Figure 5. Regulation of transcription through DNA targeting by intranuclear ROS

a | The induction of the transcription of the gene encoding vascular endothelial growth factor (VEGF) by hypoxia-inducible factor 1α (HIF1α) is enhanced through the dynein-mediated, perinuclear localization of mitochondria. Mitochondria-derived reactive oxygen species (ROS) diffuse into the nucleus, where they promote the oxidation of guanine nucleotides, forming 8-oxoguanine (8OG). **b** | Transcriptional regulation downstream of the activation of androgen receptors (ARs) or oestrogen receptors (ORs) and other nuclear receptors also involves DNA modifications by ROS. Engagement of ORs or ARs promotes the phosphorylation (P) of lysine-specific histone demethylase 1A (LSD1) by cAMP-dependent protein kinase (PKA). Active LSD1 not only demethylates histone 3 (H3) but also produces ROS, which then promote the formation of 8OG in the DNA. The altered DNA bases recruit base excision repair machinery, and the DNA breaks that are generated by 8OG DNA glycosylase 1 (OGG1) enable the activation of transcription by AR, OR and possibly also MYC.



Timeline.

A sampling of milestones in ROS biology*

CGD, chronic granulomatous disease; FDA, US Food and Drug Administration; IFNγ, interferon-γ; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species. *This Timeline is an incomplete history, with only limited citations.