Role of reactive oxygen species in cell signalling pathways

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Abstract

Reactive oxygen species (ROS) were originally thought to only be released by phagocytic cells during their role in host defence. It is now clear that ROS have a cell signalling role in many biological systems, both in animals and in plants. ROS induce programmed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signalling cascades, such as those involving mitogen-activated protein kinases.

Introduction

Reactive oxygen species (ROS) have been of interest for many years in all areas of biology. Originally ROS were recognized as being instrumental for mammalian host defence, and early work led to the characterization of the respiratory burst of neutrophils [1,2] and finally the NADPH oxidase complex [3], which is now recognized as a primary source of ROS. However, recent work has uncovered a more widespread and exciting role of ROS: that of key signalling molecules. This review will concentrate on this aspect of biologically derived ROS, and will discuss their generation and use as cellular signals. This emerging area of ROS has also been recently reviewed by others [4–6].

ROS: what are they?

ROS are species of oxygen which are in a more reactive state than molecular oxygen, and in which, therefore, the oxygen is reduced to varying degrees. For example, a primary ROS is superoxide, which is formed by the one-electron reduction of molecular oxygen (eqn 1). This is the reaction catalysed by NADPH oxidase (eqn 2, and see below), with electrons supplied by NADPH:

$$O_2 + e^- \rightarrow O_2^{-\bullet} \text{(superoxide)} \quad (1)$$

$$2O_2 + NADPH \rightarrow 2O_2^{-\bullet} + NADP^+ + H^+ \quad (2)$$

Key words: gene expression, hydrogen peroxide, MAP kinases, NADPH oxidase, signal transduction.

Abbreviations used: MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NOH, NADPH oxidase homologue; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Further reduction of oxygen produces hydrogen peroxide. This can arise from the dismutation of superoxide (eqn 3), which can occur spontaneously, especially at low pH:

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

However, this reaction can also be catalysed by a family of enzymes known as superoxide dismutase (SOD). Therefore, under physiological conditions, once superoxide is formed the presence of hydrogen peroxide becomes almost inevitable.

Further reactions may lead to the formation of hydroxyl radicals ($OH^\bullet$), especially in the presence of metal ions through the Fenton or Haber–Weiss reactions. Hydroxyl radicals are extremely reactive, with a short half-life, and will probably react with the first molecule they encounter. In neutrophils, myeloperoxidase catalyses the formation of hypochlorous acid (HOCl), while superoxide may also react with nitric oxide ($NO^\bullet$) to form another relatively reactive molecule, peroxynitrite (eqn 4):

$$NO^\bullet + O_2^{\bullet -} \rightarrow OONO^- \text{(peroxynitrite)} \quad (4)$$

It thus appears that, following the formation of superoxide anions, a cascade of ROS production is likely. Some of these ROS, especially hydrogen peroxide, are key signalling molecules, while others appear to be extremely detrimental to biological systems, effects that are dependent on the concentrations that are perceived by the cells. However, to be considered as a potential signalling molecule, ROS must: (1) be produced by a cell when stimulated to do so; (2) have an action in a cell, either the cell which produces it or a nearby cell; and (3) be removed in order to turn off, or reverse, the signal. It is now clear that some ROS, in particular hydrogen peroxide and superoxide, fulfil these criteria.

Enzymic generation of ROS: NADPH oxidase

Several enzymes are now recognized as being potentially able to produce ROS; perhaps the most important of these is NADPH oxidase.

NADPH oxidase was first discovered in neutrophils, which on stimulation undergo the respiratory burst, with the release of superoxide into the phagosome. Although the reaction that the enzyme catalyses is apparently simple (eqn 2), the enzyme itself is surprisingly complex (Figure 1) [3,7]. In resting cells, the plasma membrane contains two polypeptides, which together make up flavocytochrome b_{558}. This heterodimer, of polypeptides of 22 kDa (p22-*phox*) and approx. 91 kDa (gp91-*phox*), contains a FAD group and two haem groups, which together comprise the redox pathway, enabling the transfer of electrons from cytosolic NADPH, across the membrane, to molecular oxygen. The gp91-*phox* polypeptide is also thought to act as a H^+ channel, allowing charge compensation across the membrane [8]. However, in resting cells, no NADPH oxidase activity is seen. Other components of the enzyme reside in the cytoplasm. These cytoplasmic components are p47-*phox* (47 kDa), p67-*phox* (67 kDa) and p40-*phox* (40 kDa). On stimulation, these polypeptides are translocated to the inner face of the plasma membrane to form a fully active enzyme complex.

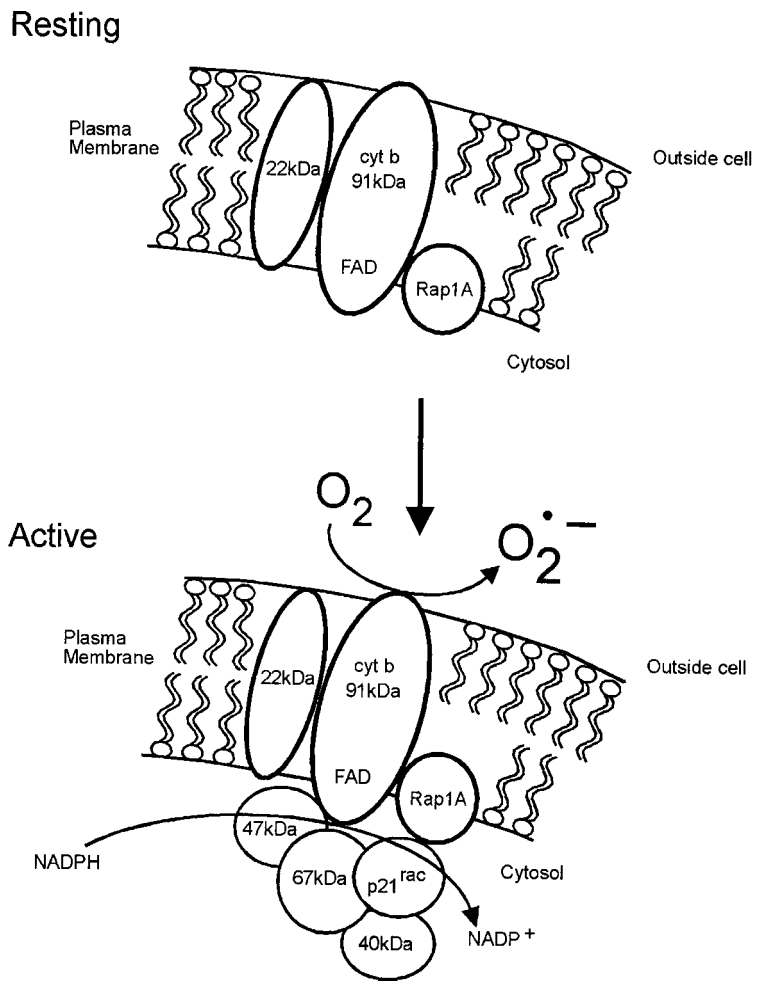
Two G-proteins are also associated with NADPH oxidase. Rap co-purifies with gp91-*phox*, although its exact role in the regulation of oxidase activity is still obscure. $p21^{rac}$ is involved in the activation of NADPH oxidase. In the inactive state, $p21^{rac}$ is in association with a GDP-dissociation inhibition factor, Rho-GDI, but on stimulation dissociation takes place, and $p21^{rac}$ translocates to the plasma membrane, where it aids in the activation of the NADPH oxidase complex.

Regulation of NADPH oxidase also involves the phosphorylation of NADPH oxidase com-

Figure 1

Schematic representation of NADPH oxidase and its activation

In order to be activated, the oxidase requires the translocation of several cytosolic components to the membrane.



ponents. PMA is commonly used to stimulate NADPH oxidase activity in cells, its action probably being mediated by protein kinase C. However, it is likely that other kinases too are involved. The modulation of intracellular calcium ions is also commonly used to activate the oxidase, and again a kinase may be involved in mediation of the calcium signal, although here direct stimulation has been suggested in some cell types. Nevertheless, it is clear that the activity of NADPH oxidase is tightly controlled [7], re-inforcing the notion that it may have a pivotal role in signalling cascades.

NADPH oxidase of non-phagocytic cells

NADPH oxidase was first characterized in white blood cells, such as neutrophils, but more recently NADPH oxidase has been found to be far more widespread, being present in cells that have no role in host defence [7]. For example, NADPH oxidase components have been reported in fibroblasts [9], mesangial cells [10], endothelial cells [11], osteoclasts [12] and chondrocytes [13,14]. Homologues of gp91-*phox* have also been cloned from plants [15,16]. However, the rate of ROS generation by NADPH oxidase in many of these cell types is very low compared with that in neutrophils, and such a disparate location for this enzyme implies a role other than simply host defence, suggesting that its proposed role in signalling is justified.

Isoforms of NADPH oxidase

Chronic granulomatous disease is characterized by a lack of functional NADPH oxidase, with the resulting impairment of the host defence function of white blood cells. The disease is inherited in an X-linked (gp91-*phox*) or autosomal fashion, dependent on the defective NADPH oxidase component, and patients are prone to repeated infection. However, Meier et al. [17] noted that, whereas neutrophils from patients with this disease had no superoxide release, fibroblasts from the same patients appeared to be normal. These researchers suggested that gp91-*phox* from fibroblasts was functionally and genetically distinct from that found in neutrophils. The first potential isoform sequence was reported from chondrocytes by Moulton et al. [18], while Bánfi et al. [19] reported the presence of three gp91-*phox* homologues in mammals: NADPH oxidase homologue-1 (NOH-1), NOH-2 and NOH-3, with alternatively spliced variants of NOH-1. Others too have reported the presence of several gp91-*phox* homologues in mammals [20,21], suggesting that the NADPH oxidase family of enzymes may have much wider roles in controlling cellular function than first thought. In plants too, homologues have been reported. For example, *Arabidopsis thaliana* has six variants [16]. As in animals, not all of the variants seem to be expressed in all cell types or tissues.

ROS from other sources

Another source of ROS is xanthine oxidoreductase or xanthine oxidase [22]. This molybdenum- and iron-containing flavoprotein catalyses the oxidation of hypoxanthine to xanthine and then to uric acid. Molecular oxygen is the oxidant, and products include superoxide and H_2O_2. This enzyme can also produce $NO^{\bullet}$, suggesting that it might have a dual role in cell signalling. Organelles containing electron transport systems, such as mitochondria and chloroplasts, may also produce ROS, although such systems are not thought to be involved in signalling processes. Finally, peroxidases are also potential sources of ROS, and it has been suggested that ROS from these enzymes are particularly important in plant tissues [23].

Signalling pathways involving ROS

In many ways, ROS are ideally suited to be signalling molecules: they are small, and can diffuse short distances; there are several mechanisms for their production, some of which are rapid and controllable; and there are numerous mechanisms for their rapid removal. Work based on the release of ROS by cells which do not have a role in phagocytosis, and where ROS have no obvious function, along with work on host defence systems in plants has led to the conclusion that ROS are key signalling molecules, although, to date, their exact mode of action still needs to be elucidated. Many studies have indicated a role for ROS in the induction or inhibition of cell proliferation, in both activation and inhibition of apoptosis, and, at higher concentrations, in the induction of necrosis [5]. Some of the biochemical effects of ROS on cells will be discussed below (Figure 2).

Induction of gene expression

Using a variety of molecular genetic techniques, several groups have now shown that the expression of a wide range of genes is regulated by hydrogen peroxide [24]. Crawford et al. [25] reported that the addition of H_2O_2 (or xanthine oxidase/

xanthine) stimulated the expression of c-*fos* and c-*myc*, and increased expression of c-*jun*, *egr*-1 and *JE* has also been reported [26,27]. Other examples include increased expression of clones identified as fibronectin and p105 co-activator in rat aorta smooth muscle cells, as shown by subtractive hybridization [28].

In our own laboratory, we have shown that in *Arabidopsis thaliana* suspension cultures, H_2O_2 induces the expression of genes encoding glutathione S-transferase, phenylalanine ammonia-lyase [29] and the plant gp91-*phox* homologue itself [15]. Further work using mRNA differential display has shown the up-regulation of other genes in *Arabidopsis*, including those encoding a protein kinase, a senescence-related protein and a DNA damage repair protein [30]. Preliminary data from screening of DNA microarrays indicate that H_2O_2 causes a more than 2-fold induction of expression of nearly 100 genes, with a more than 2-fold reduction in expression of many more (J. T. Hancock, R. Desikan and S. J. Neill, unpublished work). Further work needs to be carried out to characterize these genes and their function.

If H_2O_2 is altering gene expression patterns in cells, how is this being achieved? Transcription factors have been shown to be activated by H_2O_2. Schreck et al. [31] showed that H_2O_2 activates the transcription factor nuclear factor-κB (NF-κB). NF-κB usually resides in the cytoplasm of the cell in association with an inhibitor protein, Iκ-B, but addition of H_2O_2 to cells results in the dissociation of NF-κB from Iκ-B, and translocation of NF-κB to the nucleus. Other transcription factors affected by exogenous H_2O_2 include AP-1 (activator protein-1; a complex composed of *jun* and *fos* gene products) [32], Myb [33] and Ets [34].

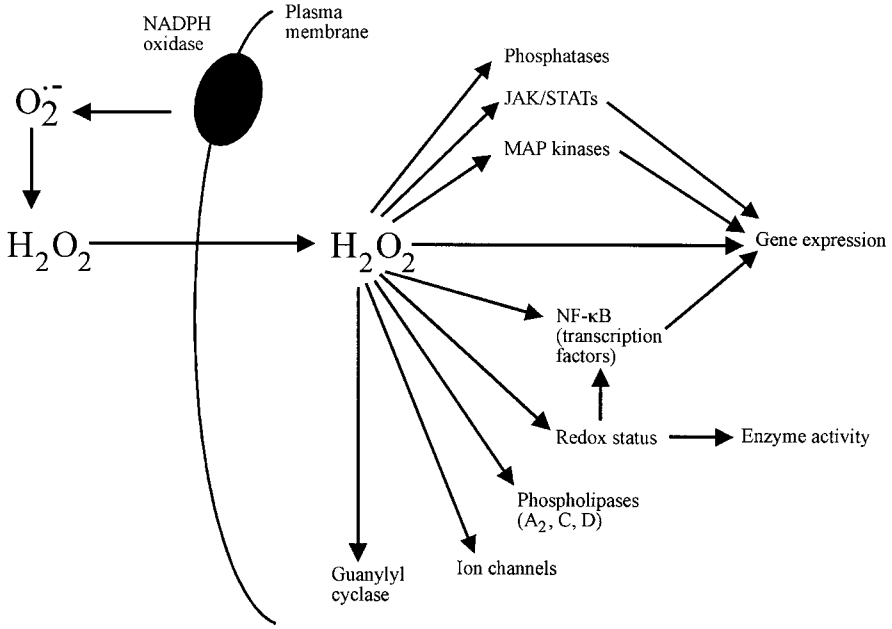
Involvement of phosphorylation

Reversible protein phosphorylation is the key biochemical event in most cell signalling pathways, and signal transduction involving ROS is no exception. Several reports have shown that mitogen-activated protein kinases (MAP kinases) are activated by H_2O_2 in both animals [35,36] and plants [37,38], which could lead to the modulation of gene expression. Whether H_2O_2 is having a direct effect on MAP kinases or activating upstream effectors needs to be established. On the other hand, H_2O_2 has also been shown to inhibit phosphatases, probably by the direct oxidation of cysteine in the active site of these enzymes [39,40]. The JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathways in

Figure 2

Potential intracellular signalling pathways that might be acted upon by hydrogen peroxide

Several signal transduction pathways lead to alterations in gene expression, while others might lead to the modulation of enzyme activities. JAK/STAT, Janus kinase/signal transducers and activators of transcription.



animal cells are also activated by H_2O_2 [41], suggesting that H_2O_2 may transduce its message into the nucleus of cells by at least two transduction pathways.

Alteration of redox status within the cell

It is now becoming apparent that the redox status inside a cell is crucial to the correct functioning of many enzymes, and can be used to alter enzyme activity; thus alteration of the redox status could act as a signalling mechanism [5]. One of the most important redox-sensitive molecules in this respect must be glutathione (GSH), which forms the GSH/GSSG couple. Changes in GSH/GSSG status have been measured after cell stimulation [42]. Certainly, H_2O_2 will have the effect of lowering the GSH content of cells, altering the redox status, and so propagation of a signal induced by H_2O_2 through this route is likely. It is suggested that enzymes such as ribonucleotide reductase and thioredoxin reductase, as well as transcription factors, might be among the targets for altered redox status [42].

Other cellular components as targets of ROS

Several enzymes which are involved in cell signalling mechanisms are also potential targets of ROS. These include guanylyl cyclase [43], phospholipase C [44], phospholipase A_2 [45] and phospholipase D [46], the latter again from direct attack of cysteine. Ion channels too may be targets [47], including calcium channels [48].

Cellular protection against oxidative damage

ROS are useful as signalling molecules and in animal and plant host defence, but on the other hand they cause cellular damage if produced in an uncontrolled manner. Therefore there is a need to remove ROS, and many enzymic and non-enzymic mechanisms are present in cells to achieve this [49].

Superoxide ions can be removed, to form hydrogen peroxide, by the enzyme SOD. The cytosolic form contains Cu and Zn (CuZn-SOD), while a mitochondrial form contains Mn (Mn-SOD) [50]. H_2O_2 can be removed by glutathione peroxidase or catalase, both of which are haem-containing enzymes. However, besides enzymes, many dietary components have antioxidant capacity, including β-carotene, ascorbate (vitamin C) and α-tocopherol (vitamin E).

Therefore, when considering how far ROS will travel, or in which part of the cell ROS will act, one has to consider that cells and organelles alike are well protected from the presence of ROS by a variety of means.

Conclusions

ROS, in particular hydrogen peroxide, are now recognized as important signalling molecules in both the animal and plant kingdoms. In animals, ROS may influence cell proliferation, cell death (either apoptosis or necrosis) and the expression of genes, and may be involved in the activation of several signalling pathways. A similar involvement is seen in plants, where ROS are particularly instrumental in host defence, inducing programmed cell death and therefore limiting the spread of invading pathogens. One of the most important enzymes for the controlled release of ROS is undoubtedly NADPH oxidase and the isoforms which have now been discovered. However, the exact action of ROS, and their interplay as signals in the face of a barrage of antioxidants, still needs to be elucidated, but will certainly be an area of intense research for the future.

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Oxidative stress in cells exposed to low levels of ionizing radiation

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Abstract

The ability of medium from γ-irradiated cells to induce early events in the apoptotic cascade, such as the mobilization of intracellular calcium, loss of mitochondrial membrane potential and increased levels of reactive oxygen species, in unirradiated cells was investigated. Medium from irradiated human keratinocytes was harvested and transferred to unirradiated keratinocytes. Intracellular calcium levels, mitochondrial membrane potential and the level of reactive oxygen species were all monitored for a period of 24 h following medium transfer. Rapid calcium fluxes (within 30 s), loss of mitochondrial membrane potential and increases in reactive oxygen species (from 6 h after medium transfer) were observed. There was no significant

Key words: apoptosis, intracellular calcium, irradiated cell conditioned medium, mitochondrial membrane potential, reactive oxygen species.
Abbreviations used: HPV, human papillomavirus; ICCM, irradiated cell conditioned medium; LET, linear energy transfer.
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