Review
Reactive oxygen species: toxic molecules or spark of life?
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Abstract

Increases in reactive oxygen species (ROS) and tissue evidence of oxidative injury are common in patients with inflammatory processes or tissue injury. This has led to many clinical attempts to scavenge ROS and reduce oxidative injury. However, we live in an oxygen rich environment and ROS and their chemical reactions are part of the basic chemical processes of normal metabolism. Accordingly, organisms have evolved sophisticated mechanisms to control these reactive molecules. Recently, it has become increasingly evident that ROS also play a role in the regulation of many intracellular signaling pathways that are important for normal cell growth and inflammatory responses that are essential for host defense. Thus, simply trying to scavenge ROS is likely not possible and potentially harmful. The 'normal' level of ROS will also likely vary in different tissues and even in different parts of cells. In this paper, the terminology and basic chemistry of reactive species are reviewed. Examples and mechanisms of tissue injury by ROS as well as their positive role as signaling molecules are discussed. Hopefully, a better understanding of the nature of ROS will lead to better planned therapeutic attempts to manipulate the concentrations of these important molecules. We need to regulate ROS, not eradicate them.

Introduction

Production of reactive oxygen species (ROS) and oxidative stress are associated with tissue injury and many pathological processes, including septic shock [1,2]. This has prompted clinical attempts to regulate oxygen radical production and oxidative stress [3-7]. Signs of oxidative stress often have been reduced, at least in blood, but by and large these clinical trials have had little beneficial outcome, although a reduction of mortality was observed in one trial [8] and a reduction in multi-organ system failure in another [9]. An underlying assumption has been that ROS randomly and indiscriminately attack important chemical pathways and, thereby, cause cell injury or death, but more recently it has become evident that ROS can act as important signaling molecules under physiological and pathophysiological conditions [10-14]. Thus, to understand the potential benefits and limitations of therapeutic approaches aimed at increasing ROS scavenging, one must understand the 'meaning' of

oxidation and ROS. It will then become evident that although ROS are potentially very toxic, they are also essential factors in normal metabolism.

Oxygen is now the most prevalent element in the earth's crust [15]. It exists in air as a diatomic molecule, O_2. Except for a small number of anaerobic bacteria, all living organisms use O_2 for energy production and it is thus essential for life as we know it. Energy production from food material by organisms requires 'oxidation', which means the loss of electrons. In anaerobic organisms, electrons are taken up by hydrogen, but in aerobic organisms, the loss of electrons occurs much more efficiently through the use of electron carriers such as nicotinamide adenine dinucleotide (NAD+) and flavins, which are 'reduced' in the process by gaining electrons from target molecules and are re-oxidized by donating electrons to O_2 through oxidative phosphorylation. The potential for O_2 to oxidize other molecules also makes it toxic. Oxidation is the basic process in combustion; fires do not burn without O_2. It is also the cause of rust. Oxidation can inactivate important enzymes and anaerobes that do not have anti-oxidant mechanisms do not survive in an O_2 environment. Thus, for organisms to have evolved in an O_2 world there has had to be evolution of potent mechanisms to control oxidative processes.

Terminology

Before continuing with a discussion of potential beneficial and harmful aspects of ROS, we need to review the terms involved [15]. Oxidation is the gain of oxygen by a substance or a loss of an electron. A useful reminder is 'LEO', which stands for 'lose electron oxidized'. Reduction is the loss of oxygen by a substance, the gain of an electron or the gain of hydrogen; a useful reminder is 'GER', which stands for 'gain electron reduced'. An oxidizing agent takes an electron or hydrogen from another chemical or adds oxygen. A reducing agent supplies electrons or hydrogen to another chemical, or removes oxygen. An important chemical principle is that because of their spin, electrons are most stable when they are paired in their orbits. Unpaired electrons are attracted to

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PTP-1B = phosphatase 1B; ROS = reactive oxygen species; SERCA = sarco/endoplasmic reticulum calcium ATPase; SOD = superoxide dismutase.

magnetic fields, which makes them more reactive. Substances that have unpaired electrons and are capable of independent existence are called free radicals. By this definition, atomic hydrogen is a free radical because it only has one electron. O_2 is a radical because it has two unpaired electrons in its outer orbitals, and this gives O_2 its reactivity. However, the two unpaired electrons on O_2 have parallel spins, which means that O_2 can only oxidize another molecule by accepting a pair of electrons that have antiparallel spin so as to fit into the two vacant spaces of O_2. This tends to make O_2 only accept one electron at a time and thus react sluggishly with non-radicals. Thus O_2 is the most stable state of oxygen. Superoxide ($O_2^{\bullet-}$) has one more electron than O_2. Since only one electron is unpaired in $O_2^{\bullet-}$, it is simpler for it to accept an electron and is thus more reactive than O_2. However, $O_2^{\bullet-}$ is still not a very reactive radical; in the presence of H^+ or $HO_2^{\bullet}$ it can reduce $O_2^{\bullet-}$ to H_2O_2 or be oxidized to O_2.

Another term that is often used is 'reactive oxygen species' (ROS). This term includes radicals as well as chemicals that can take part in radical type reactions (i.e. gain or loose electrons), but are not true radicals in that they do not have unpaired electrons. Examples of non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1\Delta gO_2$). An important product of the two radicals $O_2^{\bullet-}$ and NO is peroxynitrite ($ONOO^-$); this reaction occurs at a diffusion limited rate [16,17]. Although not a radical itself, $ONOO^-$ can result in cytotoxic processes, including lipid peroxidation, the formation of nitrotyrosine residues that can inactivate enzymes, depletion of glutathione, and DNA injury. Besides oxygen-based radicals, there are also reactive nitrogen species such as nitric oxide (NO) and nitrogen dioxide (NO_2), sulfur based molecules such as thinyl (RS) and perthinyl (RSS), as well as carbon centered molecules such as trichloromethyl ($CCl_3^{\bullet}$), which is a product of metabolism of carbon tetrachloride (CCl_4) [15].

Sources of $O_2^{\bullet-}$ and ROS

Under the conditions of normal metabolism the most important source of $O_2^{\bullet-}$ is the mitochondrial electron transport chain, which leaks a few electrons directly onto O_2 as part of normal metabolism. It is estimated that 1% to 3% of O_2 reduced in mitochondria is in the form of $O_2^{\bullet-}$ [18]. This comes from two sites, complex 1 (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase), with the latter being the major source under normal conditions [11].

Several enzymes also contribute to $O_2^{\bullet-}$ production. One of the best characterized is xanthine oxidase, which is present in the cytosol of many tissues but also can be found in circulating blood and bound to glycosaminoglyan sites in the arterial wall [19]. Normally the enzyme acts as a dehydrogenase and transfers electrons to NAD+ rather than O_2, but in ischemia reperfusion [20,21] or in sepsis [21,22] the active site of the enzyme is oxidized and the enzyme acts as an oxidase and produces $O_2^{\bullet-}$.

In phagocytic cells the major source of $O_2^{\bullet-}$ is a multi-component oxidase called NAD(P)H oxidase [23,24]. In response to membrane signals this complex produces a burst of $O_2^{\bullet-}$ that is important for killing invading microorganisms. Genetic mutations in components of the complex result in chronic granulomatous disease, which is characterized by repeated infections. There are at least five components to the complex. Two, p22phox (phox stands for phagocyte associated oxidase) and gp91phox (subsequently called NOX2) are found in membranes [25,26]. NOX2 is the component that produces $O_2^{\bullet-}$. The complex is activated when the cytosolic component p67phox is transported to the membrane complex by the transporter molecule p47phox [27]. The attachment of p67phox to the membrane complex results in a conformational change in p22phox that exposes the active site on NOX2. The small g-protein Rac also contributes to the activity of the enzyme and transmits membrane signals to the complex. Recently, a family of non-phagocytic NOXs with the same basic components as the phagocytic type have been identified in numerous types of cells, including vascular smooth muscle, endothelial, skeletal muscle, fibroblast, and mesangial cells [28-30]. The non-phagocytic form produces much lower amounts of $O_2^{\bullet-}$ compared to the phagocytic form but is constitutively active.

$O_2^{\bullet-}$ is also produced by a number of metabolically active enzymes as part of their normal function or when there is inadequate substrate. For example, cytochrome P450 enzymes can produce $O_2^{\bullet-}$ as a side reaction when they breakdown target molecules [15]. Nitric oxide synthases, the family of enzymes that produce NO, produce $O_2^{\bullet-}$ when the substrates L-arginine or co-factor tetrahydropteridines are insufficient [21,31,32].

$O_2^{\bullet-}$ can also be produced by cyclooxygenase as part of arachidonic acid metabolism. $O_2^{\bullet-}$ even can be produced through auto-oxidation of molecules such as gylceraldehyde, FMNH2, FADH2, adrenalin, noradrenalin, dopamine and thiol containing molecules such as cysteine in the presence of O_2 [1,15]. Since we live in an oxygen rich environment, and ROS are byproducts of normal metabolism, potent protective mechanisms have evolved to allow life to continue. One of the most fundamental antioxidant enzymes is superoxide dismutase (SOD), which catalyzes the reaction of two $O_2^{\bullet-}$ and two H^+ to H_2O_2 (reduced) and O_2 (oxidized) [33]. There are three forms to this enzyme: SOD1, a copper/zinc (Cu/Zn) isoform present in the cytosol; SOD2, a manganese (Mn) isoform present in mitochondria; and SOD3, a Cu/Zn isoform present in the extracellular space. Knockout of SOD2 in mice is lethal in the first week of life [34,35] whereas deficiencies of SOD1 and SOD3 are not lethal but result in less tolerance of neuronal injury [36] or hyperoxia, respectively [37]. H_2O_2 itself is not a radical but is a ROS and may actually account for most of the $O_2^{\bullet-}$ reactions. What makes H_2O_2 so important is that it is more stable than $O_2^{\bullet-}$ and can diffuse across membranes. In the presence of iron in the ferrous form

(Fe^{2+}), H$_2$O$_2$ can be reduced to the highly reactive OH$^\bullet$ radical. It is thus important that H$_2$O$_2$ also be reduced in a controlled manner and this is achieved by catalase or glutathione peroxidase. Other antioxidants include cysteine, glutathione itself, ascorbic acid (vitamin C) and α-tocopherol (vitamin E), which can also scavenge peroxynitrite.

Production of injury by ROS

One of the major toxic effects of excessive ROS is damage to cellular membranes by the process of lipid peroxidation. Species such as OH$^\bullet$, HO$_2$$^{\bullet-}$, and OONO$^-$, but not O$_2$$^{\bullet-}$, can extract an H from methylene (-CH$_2$-), which creates the carbon radical -$^\bullet$CH-. This carbon radical then attacks other -CH$_2$- groups in lipid molecules, and creates a chain reaction that alters the fluidity and shape of the membrane. This is the same process that makes oil rancid. A consequence of the change to the cell membrane is disruption of calcium handling, which is essential for intracellular signaling. Lipid peroxides can also damage DNA and proteins.

Attack of DNA by ROS results in DNA strand breaks. As with lipids, O$_2$$^{\bullet-}$ and H$_2$O$_2$ do not do this by themselves but do so in the presence of hypochlorous acid (HOCl). It is also possible that oxidative stress results in the release of bound intracellular iron and copper ions that can then generate the highly toxic OH$^\bullet$ through what is known as the Fenton reaction [15]. The potential for this to produce mutations and to alter normal transcriptional and translational processes is obvious. Besides these direct effects of oxidative injury, there can be indirect injury because the nicks and breaks in DNA strands can trigger activation of Poly(ADP) polymerase (PARP), which alters gene expression, DNA replication and may trigger apoptosis. It can also deplete NAD+, which leads to cellular ATP depletion [38].

Proteins, too, can be targets of oxidative alterations. Protein oxidation disrupts receptors, enzyme function and signal transduction pathways. The amino acid tyrosine is particularly prone to attack by ROS, especially reactive nitrogen species such as OONO$^-$ [39]. The product of OONO$^-$ and tyrosine is 3-nitrotyrosine and antibodies against it are used as a 'footprint' of protein oxidation [40]. Oxidation of proteins also can lead to products with carbonyl groups [41]. The amino acids histidine, arginine, lysine and proline are especially vulnerable. However, just because a protein is oxidized does not mean that it has lost its function and the biological significance of oxidation of a particular protein needs to be confirmed by evidence of an alteration in function. An example of a protein function altered by oxidation is the inactivation of the intra-mitochondrial SOD, SOD2, by peroxynitrite [42]. Because O$_2$$^{\bullet-}$ scavenging is reduced, oxidative processes are accelerated. Potentially important functional sites for oxidation of proteins are the -SH groups because the formation of -S-S- bonds between different protein strands or parts of the same strand can result in conformational changes in the protein that alter its function (see below).

ROS in sepsis

There is evidence from animal studies that an increase in ROS in sepsis is of pathophysiological importance. Oxygen radical scavengers reduce lung injury in animal models [43-48] and improve hemodynamics [48,49]. An interesting and potentially clinically important example of O$_2$$^{\bullet-}$ induced injury is the deactivation of catecholamines in inflammatory reactions [50]. Catecholamines can act as antioxidants because of their ability to interact with ROS, but this process also leads to their deactivation and the formation of adrenochromes, which are toxic themselves. Of interest, in the first identification of SOD, one of the tests of the activity of the enzyme was the prevention of oxidation of catecholamines [33]. The potential clinical importance of the oxidation of catecholamines was demonstrated by Salvemini and coworkers [50] who showed that ROS decrease the activity of catecholamines and oxygen radical scavengers restore cardiovascular responsiveness to catecholamines in an animal model of sepsis.

There is also evidence for a clinically significant role for ROS in humans. Patients with sepsis who are able to achieve a normal antioxidant potential in their plasma have better survival [51] and treatment of septic patients with the antioxidants glutathione and N-acetylcysteine decreases measures of oxidative injury [4]. N-acetylcysteine reduces the respiratory burst from neutrophils of septic patients [52] and patients with lung injury randomized to antioxidant therapy with N-acetylcysteine versus placebo had an improvement in systemic oxygenation and a reduction in the need for ventilatory support [5]. An improvement in hepatic blood flow in septic patients has also been observed [6]. On the other hand, no significant clinical advantage to the administration of N-acetylcysteine was observed in two studies [3,7]. An important limitation of N-acetylcysteine is that it works by increasing the intracellular cysteine concentration, which normally is high relative to the plasma concentration [53]. Thus, potentially toxic plasma levels are needed to reach the necessary intracellular levels. N-acetylcysteine also has a low Km for the removal of O$_2$$^{\bullet-}$, which is why it has to be present at high concentrations. Augmenting oxygen radical scavenging activity in patients with septic shock by combining N-acetylcysteine and glutathione produced a trend towards less organ damage [4] but results were not conclusive. To date there is no clear evidence that antioxidant therapy alters outcome in septic patients [54], although as noted in the introduction, supplementation of feeds with vitamins was shown to reduce mortality of a general group of severely ill patients [8] and reduce multiorgan dysfunction in a group of critically ill patients who were primarily trauma victims [9].

ROS and cell signalling

Perhaps the failure to find a clinical role for therapies aimed at the reduction of ROS is that they are based on the limited paradigm that ROS only cause injury. An alternative view is

that although ROS are potentially highly toxic, redox reactions are also part of the basic chemical processes of life [10,11]. Since organisms have had to develop efficient regulatory mechanisms to keep the production of ROS under control, these same mechanisms could be used to regulate other intracellular processes [12-14,53,55]. A parallel might be seen with that of Ca^{2+} handling. The intracellular Ca^{2+} concentration is kept at less than 1/10,000 of extracellular Ca^{2+} so as to avoid the interaction of Ca^{2+} and phosphate and bone formation. Because of the large transmembrane gradient of Ca^{2+}, the leak of small amounts of Ca^{2+} across cell membranes through specialized channels can provide one of the cell's basic signalling mechanisms. Similarly, regulation of extracellular and intracellular levels of $O_2^{\bullet-}$ and H_2O_2 could provide potential for signalling of extracelluar to intracellular mechanisms. In this paradigm, ROS are not just random destructive species but regulators of metabolic processes and part of the chemistry of life [10,11]. Further-more, evidence of oxidative injury may be the end result of the inflammatory process rather than the major cause of injury, in which case the use of antioxidants may be too late. Another analogy might be helpful. Consider walking along a beach and observing a rusted old ship lying on the shore. You conclude that the reason why the ship was abandoned is because it is so rusted (oxidized) until you walk past the ship and notice a large hole in the hull. You then realize that the ship was abandoned because of the hole and rusted when it was no longer cared for. Signs of oxidative changes may simply indicate that molecules or cells have been abandoned by the organism and are not themselves the major cause of the disease process.
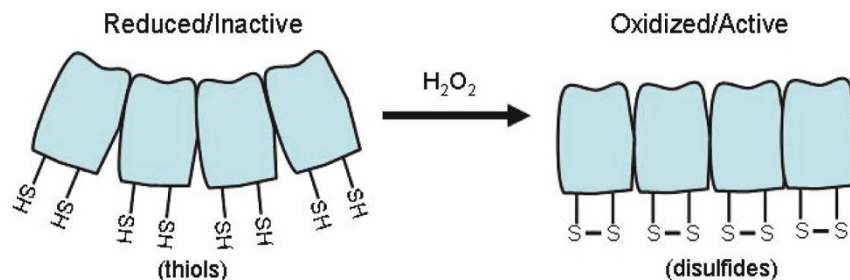
Although there is a lot of evidence indicating that ROS and the redox state have a signaling role in bacteria and plants, there was less evidence in mammalian cells until recently. For example, in bacteria the transcription factor OxyR is redox sensitive [13]. There is now an increasing number of examples in animals of ROS-based signaling, including protein tyrosine phosphatase 1B (PTP-1B) [56], thioredoxin [57], SERCA2 [58] and Ras [59]. A well-characterized radical that has a major role in normal physiological function is nitric oxide (NO$^{\bullet}$). This radical has a central role in the regulation of vascular tone, nerve function and immune regulation. Even the potentially toxic by-product of NO$^{\bullet}$ and $O_2^{\bullet-}$, OONO$^-$, has recently been shown to play a role in the regulation of vascular tone [58]. Cohen and coworkers found that NO$^{\bullet}$ induced dilatation occurs by the production of low concentrations of OONO$^-$, which directly stimulates the sarco/endoplasmic reticulum calcium (Ca^{2+}) ATPase (SERCA) to decrease intracellular Ca^{2+} and thereby produce vasodilatation. This occurs by reversible S-glutathiolation of the thiol of a cysteine molecule on SERCA. Thus, by removing $O_2^{\bullet-}$ and preventing the formation of OONO$^-$, superoxide scavengers actually blocked NO-induced vascular relaxation. However, high levels of oxidative stress, including high concentrations of OONO$^-$, resulted in irreversible oxidation of key thiols and prevented normal NO-induced relaxation. An important lesson may be learnt from the NO system. Endothelial and neuronal cells that use NO for signalling produce NO in small amounts, whereas macrophages and neutrophils that use NO to attack invading organisms produce large amounts. Similarly, the NAD(P)H oxidase in phagocytic cells produces large quantities of $O_2^{\bullet-}$, whereas the NAD(P)H oxidases in non-phagocytic cells produce much smaller amounts of $O_2^{\bullet-}$, consistent with a signalling role.

The role of ROS in the signaling of a number of growth factors has also been well established. An excellent example is the role of ROS in angiotensin signaling as established by Griendling and co-workers [29,60-62]. They showed that exposure of vascular smooth muscle to angiotensin II results in smooth muscle growth that is dependent upon increased production of $O_2^{\bullet-}$ by NAD(P)H oxidase and its subsequent dismutation to H_2O_2. H_2O_2 then activates downstream prosurvival pathways and, *in vivo*, this results in vascular hypertrophy. Other growth factors such as platelet derived growth factor have been shown to have similar signaling mechanisms [63].
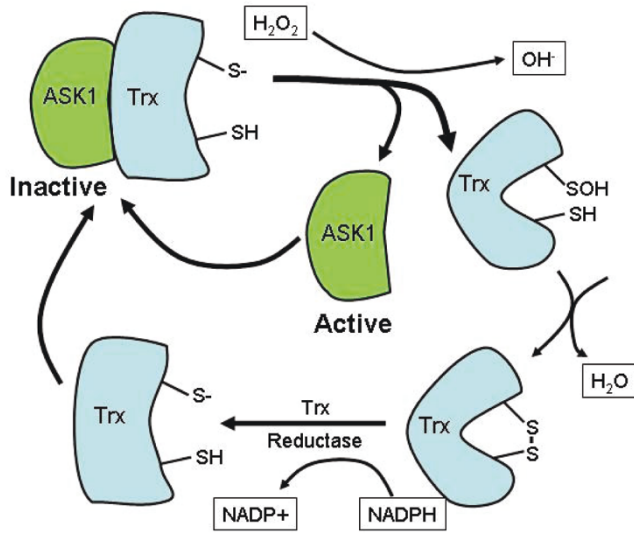
ROS also play a role in the intracellular signaling of tumor necrosis factor-α [22,64-72] and this too seems to occur

Figure 1

The change from thiols (-SH) to disulfide bonds (-S-S-) can produce a conformational change that may allow better protein-protein or protein-DNA interactions. Adapted from Droge *et al.* [11].
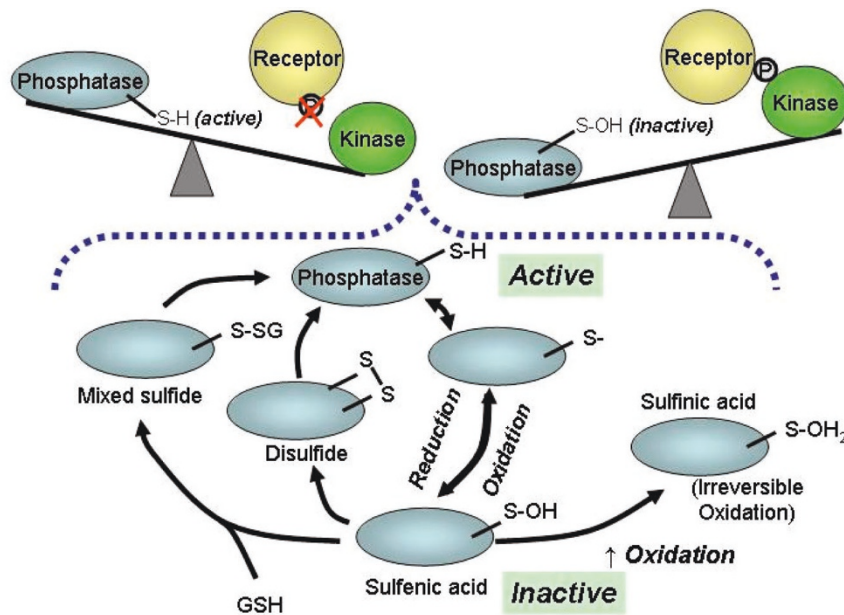
Figure 2

Oxidation of thioredoxin (Trx) by hydrogen peroxide (H_2O_2) leads to a change in shape of the molecule and the release of the transcriptional factor ASK1. Trx is then reduced again by Trx reductase, which allows it to again bind to ASK1 and inactivate this transcriptional factor. Through this mechanism the redox state of the cell can regulate the activity of the transcriptional factor ASK1.

through $O_2^{\bullet-}$ produced by NAD(P)H oxidase and likey involves regulation of the transcriptional activity of NFκB. Similarly, it has recently been shown that lipopolysaccharide activation of Toll-like receptor 4 increases $O_2^{\bullet-}$ production by NAD(P)H oxidase and this too leads to NFκB activation [73].

Various mechanisms have been explored recently that can explain how ROS can signal intracellular events. These generally involve the oxidation of cysteine residues and formation of -S-S- bonds [12,14,53,74,75] (Figs 1 to 3). These bonds can be within a molecule and result in a conformational change (Fig. 1) or between protein strands, in which case they result in dimerization of proteins. The creation of -S-S- bonds can also result in the release of an inhibitory molecule (Fig. 2). Some reactions are irreversible and result in protein instability or irreversible protein cross-linking. However, an interesting reversible process is oxidation of cysteinyl thiols by S-glutathiolation from thiol disulfide exchange reactions involving oxidized glutathione or from direct oxidation of protein cysteinyl thiols followed by reaction with reduced glutathione [75] (Fig. 3). In the case of PTP-1B, stabilization of an oxidized cysteine occurs through the formation of a mixed disulfide with glutathione (Fig. 3). The formation of the mixed disulfide prevents the irreversible oxidation of the thiol to sulfinic or sulfonic acid and allows for the reactivation of the enzyme by cellular thioreductase,

Figure 3

Regulation of phosphatase by the redox state. Cysteine molecules have sulfur atoms (S) that are protonated and not reactive in most proteins. However, on some molecules, such as phosphatases, S can form thiolates (S-) at normal pH and these can be reversibly oxidized. The top of the figure shows the balance between phosphatase activity (which dephosphorylates molecules) and kinase activity (which phosphorylates and activates molecules). Phosphatase activity is regulated by the redox state as shown in the cycle below the bracket. Oxidation to sulfenic acid (-S-OH) is reversible. This can occur by glutathiolation (GSH) or by the formation of disulfides. However, excessive oxidation leads to sulfinic acid, which cannot easily be converted back to reduced forms of sulfur.

Table 1

A check list for the evaluation of the utility of anti-oxidant therapies

1	What is the reactive oxygen species that is causing the oxidative injury?
2	What is the target molecule?
3	What is the source of the ROS?
4	What types of cells produce the ROS?
5	Where in the cells are the ROS produced?
6	What is the potentially useful role of the ROS?

although recently it has become apparent that even sufinic groups can be re-oxidized [76-78].

Implications

ROS are an essential part of many metabolic pathways; they are part of the flame of basic energy producing processes. Organisms have had to evolve elaborate mechanisms to live with these reactive molecules and seem also to have evolved to use the reactive nature of these molecules for intracellular signal transduction. Thus, a key concept in dealing with ROS must be to regulate but not eradicate, for turning off production of ROS is tantamount to turning off the engine that powers us. ROS also seem to have specific roles in different cell types and thus therapeutic strategies for the manipulation of ROS should take into account the source of ROS, the targets of the ROS, specific cell types involved and the specific location of ROS production in these cells, for one needs to know that the potential therapeutic agent actually can get to the site of excess ROS production. A list of things to consider when examining the potential of a therapeutic agent to deal with ROS is given in Table 1. In the management of ROS we will need to be careful to not repeat the mistake that was made with global inhibition of NO production.

Competing interests

The author(s) declare that they have no competing interests.

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