Minireview

Nitric oxide and oxygen radicals: a question of balance

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Abstract The production of superoxide and nitric oxide individually has been associated with the development of several diseases but only recently has it been realised that interactions between them may also be important in disease pathology. The central hypothesis which is emerging is that the balance between nitric oxide and superoxide generation is a critical determinant in the aetiology of many human diseases including atherosclerosis, neurodegenerative disease, ischaemia-reperfusion and cancer. These ideas are discussed in this short overview and placed in the context of the current and future status of therapies which could modulate the balance between nitric oxide and superoxide.

Key words: Nitric oxide; Superoxide; Reactive nitrogen species; Peroxynitrite; Oxygen radicals

1. Introduction

Oxygen and nitrogen together comprise over 98% of the air we breathe. Despite being essential for most forms of life, the high content of oxygen in the atmosphere means that oxidation reactions are commonplace in our environment. Although our body uses oxygen and oxidation reactions to good effect for generating energy and killing invaders, we cannot avoid some of the unwanted side reactions, which are caused by partially reduced forms of oxygen and result in the physiological equivalents of rusting and rancidity. In recognition of the important role that oxygen-derived species play in biology, they have often been grouped together and called reactive oxygen species (ROS) [1]. This global term includes both oxygen radicals and non-radical derivatives of oxygen (Table 1). Many ROS can modify essential biological molecules such as lipids, proteins and DNA, leading to changes in function.

Nitrogen also plays a central role in biology and is incorporated into all of the major classes of biomolecules. However, it is only recently that a physiological role for reactive nitrogen species (RNS: Table 1) has been appreciated following the discovery that the simplest compound of nitrogen and oxygen, nitric oxide ($NO^{\bullet}$) is produced in vivo. Nitric oxide has an astounding range of biological roles including modulation of vascular tone, memory formation and inflammation [2–6]. NO often achieves these effects by binding to the haem group of the soluble form of the enzyme guanylate cyclase, but rarely through irreversible chemical modifications of other molecules. However, one important function of $NO^{\bullet}$ is thought to be in the macrophage-dependent killing of parasites, and possibly cancer cells, indicating the potential of this free radical to mediate cytotoxic and pathological effects [5].

Here we describe an emerging picture which suggests that the interactions between RNS and ROS generate potentially cytotoxic agents which may mediate some of the pathology associated with Parkinson's disease, chronic inflammation, atherosclerosis and cancer. Some of the complex chemical interactions between ROS and RNS which have already been described in the literature are shown in Fig. 1.

It is evident from this scheme that some RNS may normally be useful but can be toxic in excess. Nitric oxide is the prime example. Other RNS such as nitrogen dioxide ($NO_2^{\bullet}$) and peroxynitrite ($ONOO^-$) are probably always damaging. The same appears to be true for ROS; superoxide ($O_2^{-\bullet}$) is often useful but the hydroxyl radical (OH) is probably always bad. An added complexity is that some of the endogenous defence mechanisms against the damaging RNS may lead to the generation of potentially cytoprotective species such as the nitrosothiols (RSNO in Fig. 1). However, it is evident that the *balance* between $NO^{\bullet}$ and ROS (especially $O_2^{-\bullet}$) at sites of injury can affect the net outcome, and may, therefore, be a key determinant of whether the result of tissue injury is resolution and repair, or chronic inflammation.

2. RNS/ROS interactions

Levels of $NO^{\bullet}$ in excess of that required to activate guanylate cyclase can inhibit glycolysis, the mitochondrial respiratory chain and DNA replication [5,7–13]. These effects can be direct, eg inhibition of ribonucleotide reductase [10] and mitochondrial cytochrome *c* oxidase [7] but often they involve $NO^{\bullet}$ interactions with ROS. Nitric oxide reacts very rapidly with oxygen radicals. Thus $NO^{\bullet}$ reacting with $O_2^{-\bullet}$ generates $ONOO^-$. This species may mediate several of the cytotoxic effects of $NO^{\bullet}$, such as the destruction of FeS centres in enzymes. Persistent blockade of cytochrome *c* oxidase by $NO^{\bullet}$ may lead to the release of free calcium ions from the mitochondrial matrix into the cell cytosol [12]. Nitric oxide also reacts with lipophilic peroxyl radicals, important propagating species in the biological chain reaction of lipid peroxidation, to generate alkyl peroxynitrates (LOONO). These appear far more stable than $ONOO^-$ [13]. If LOONO derivatives can be metabolised without the release of toxic free radicals then the reaction of $NO^{\bullet}$ with peroxyl radicals is potentially beneficial because it allows $NO^{\bullet}$ to stop lipid peroxidation. The ratio of $NO^{\bullet}$ to ROS may be all important since $ONOO^-$ can cause lipid peroxidation. Thus, a 1:1 ratio of $O_2^{-\bullet}$ to $NO^{\bullet}$ generates $ONOO^-$ and induces

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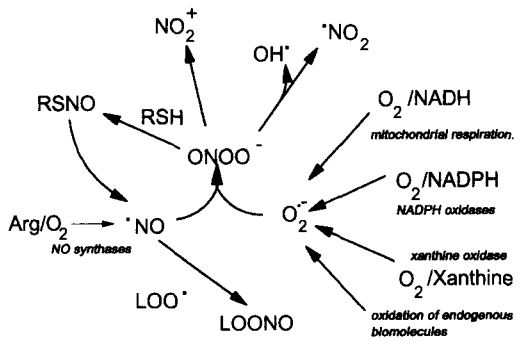

Fig. 1. Origins and interactions of ROS and RNS in biological systems. The most important route for the formation of RNS is the oxidation of arginine by NO• synthases (NOS) which are essentially of 2 types. These are the constitutive calcium/calmodulin-dependent forms (cNOS), which produce low levels of NO•, and inducible (iNOS) forms which have tightly bound calmodulin, are permanently active, and capable of generating high levels of NO•. The major sources of O_2^{-}• in vivo, are shown and include; mitochondrial respiration, NADPH oxidases; a family of enzymes which catalyse the vectorial synthesis of O_2^{-}• from oxygen, the enzyme xanthine oxidase which catalyses the formation of O_2^{-}• and H_2O_2 from oxygen and xanthine or hypoxanthine. The different RNS that may directly contribute to the pathogenesis of disease are ($ONOO^-$; peroxynitrite, NO_2^+; the nitronium cation which results in nitration of tyrosine residues, and $NO_2^•$, which may mediate a number of pro-oxidant reactions including nitration). Those RNS with potentially beneficial effects include NO• itself, the adducts formed from the termination reaction of NO• with peroxyl radicals (LOONO) and nitrosothiols (RSNO) formed from the reaction of peroxynitrite with thiols). Nitric oxide reacts directly with oxygen to form $NO_2^•$ but this third-order reaction is very slow at physiological concentrations of NO• and ultimately yields nitrite [38].

lipid peroxidation whereas an excess of NO• can inhibit lipid peroxidation by scavenging peroxyl radicals [13].

3. Relevance to human disease

3.1. Cardiovascular disease

Vascular endothelial cells generate NO• for a multitude of physiological reasons and they may also secrete O_2^{-}• [2,14]. Hence $ONOO^-$ (Fig. 1) can form in the vasculature. Phagocytes adhering to vascular endothelium can also produce RNS and ROS and interactions between them may have regulatory significance, e.g. NO• inhibits platelet and phagocyte adhesion to the endothelium [2,6]. In advanced atherosclerotic lesions thrombosis plays an important role and this normally beneficial biological process is controlled in part by the NO•-dependent inhibition of platelet aggregation [2]. However, in atherosclerotic lesions excess production of O_2^{-}• may cause loss of the modulatory action of NO• and at the same time yield $ONOO^-$ which is pro-aggregatory and so could commit platelets in this environment to thrombus formation [15].

In inflammation the balance between NO• and O_2^{-}• is altered during the interplay of the numerous mediators released in this process, but it must return to normal for resolution and healing. Failure to normalise may be an important factor predisposing to chronic inflammation. Atherosclerosis may be one example where this occurs. At an early stage in the development of this disease, chemical transformation of low density lipoprotein (LDL) occurs in the vessel wall [16]. Lipid peroxidation is one way in which LDL modification occurs and it results in the formation of a rancid mixture of toxic products. Probably in an attempt at protection, modified LDL is engulfed by macrophages; the resulting lipid laden foam cells may then become part of the problem because of the effect of their secretory products on other cells in the lesion, e.g. encouraging smooth muscle cell proliferation [16]. We do not really know how oxidation of LDL begins in the artery wall. However, peroxynitrite could be involved since it efficiently oxidises LDL [17,18], causes a rapid depletion of several antioxidants (ascorbate, urate, protein thiols and ubiquinol) [19] and releases copper ions from the plasma protein caeruloplasmin [20]. Copper ions are powerful catalysts of LDL oxidation which have been detected in advanced human atherosclerotic lesions [21,22]. Consistent with formation of $ONOO^-$ in the vasculature, immunoreactive material which cross-reacts with an antibody recognising nitrotyrosine (Fig. 2) has been found in human atherosclerotic lesions [23].

The changes which occur during atherosclerosis also include loss of the primary function of the vessel in acting as a conduit of variable size for blood. Early in hypercholesterolaemia a profound endothelial dysfunction appears; the responses to the normal vasodilatory effects of NO• are lost through a shift in the balance between NO• and O_2^{-}• towards the latter [14,18]. As predicted from this hypothesis the O_2^{-}• scavenging enzyme superoxide dismutase (SOD), diminishes this endothelial dysfunction, as does the substrate of the NO• synthases, L-arginine [18,24]. If the balance between the generation of NO• and O_2^{-}• in the vasculature can be 'reset' with either arginine or SOD, one would expect that decreasing NO• synthesis would be pro-atherogenic, as has been reported [25]. Furthermore, it has

Table 1
Reactive oxygen species (ROS)

Radicals	Non-radicals
Superoxide, O_2^{-}•	Hydrogen peroxide, H_2O_2
Hydroxyl, OH•	Hypochlorous acid, HOCL*
Peroxyl, $LO_2^•$	Ozone, O_3
Alkoxyl, LO•	Singlet oxygen $^1\Delta g$
Hydroperoxyl $HO_2^•$	Lipid peroxides
Reactive nitrogen species (RNS)	
Radicals	Non-Radicals
Nitric oxide**, NO•	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^•$	Dinitrogen trioxide N_2O_3 Dinitrogen tetroxide N_2O_4 Peroxynitrite, $ONOO^-$
	Alkyl peroxynitrites LOONO

A free radical is any species capable of independent existence containing one or more unpaired electrons; it is denoted by a superscript dot. Reactive is a relative term: NO• and O_2^{-}• are poorly reactive whereas OH• reacts with everything. The other species have intermediate reactivities. NO• and O_2^{-}• are similar in other respects; they both have important physiological roles but are toxic in excess, often by generating other oxidants. Ironically, O_2^{-}• is one of the few molecules with which NO• reacts quickly (and vice versa).
*Could equally well be called a 'reactive chlorine species'. **The correct chemical name for nitric oxide is nitrogen monoxide. However, common usage generally prevails in these matters as a brief search on Medline with these two terms will soon confirm.

Fig. 2. Detection of RNS-induced damage detection of short-lived and reactive species such as many of the RNS is difficult but some of the reaction products are stable. One important example is nitration of aromatic amino acids, which occurs by their reaction with $ONOO^-$, probably via $NO_2^{\bullet}$ and NO_2^+. Tyrosine is especially sensitive, giving a nitrotyrosine adduct which can be measured by HPLC [19,32] (directly or after reduction to aminotyrosine, which can be detected electrochemically, giving increased sensitivity), by GC/MS or by antibodies directed against nitrated proteins [23]. Nitrotyrosine is excreted in human urine and as such might be useful as a total body marker of RNS [54]. Other potential markers for RNS include nitrate/nitrite, nitrosothiols, oxidation of oxyhaemoglobin, depletion of antioxidants, deaminated DNA bases, 8-nitroguanine and the formation of end-products of the reactions of RNS with $LO_2^{\bullet}$ and $RO^{\bullet}$ during lipid peroxidation, such as LOONO (Fig. 1).

recently been shown that oxidised LDL decreases the expression of endothelial $NO^{\bullet}$ synthase [26]. It is attractive to hypothesize that the loss of endothelial $NO^{\bullet}$ production plays a role in phagocyte adhesion and activation at sites of injury, as occurs after ischaemia-reperfusion [27].

3.2. Protective mechanisms

Several antioxidants can scavenge $ONOO^-$ [19]. The interactions with thiols is of particular interest: $ONOO^-$ can irreversibly oxidise them to higher oxidation states, but nitrosothiols can also form which may later act as $NO^{\bullet}$ donors [15,28]. Indeed, when isolated vascular tissues are exposed to $ONOO^-$, vasorelaxation occurs by a mechanism characteristic of release of $NO^{\bullet}$ from a 'carrier molecule' such as a nitrosothiol [28,29]. Repeated exposure to $ONOO^-$ results in a progressive decrease in the efficiency of the vasorelaxing effect and inhibition of the ability of the heart to respond to other vasorelaxing agents such as isoproterenol or prostacyclin [29]. These data suggest that $ONOO^-$ may overwhelm thiol-dependent protective mechanisms, leading to $ONOO^-$-dependent vascular dysfunction. Another potential protective mechanism would be the removal of $O_2^{-\bullet}$ by the enzyme superoxide dismutase (SOD). However, the rate of reaction between $NO^{\bullet}$ and $O_2^{-\bullet}$ is so fast that is is effectively only limited by the rate of diffusion of the two radicals. For SOD to be effective it must, therefore, be at a concentration comparable to or higher than $NO^{\bullet}$ and close to the site of $NO^{\bullet}/O_2^{-\bullet}$ generation. Control of the $O_2^{-\bullet}/NO^{\bullet}$ reaction may be the function of the 'extracellular' SOD enzymes which bind to endothelial cell surfaces and the arterial intima [30].

If the $O_2^{-\bullet}/NO^{\bullet}$ balance is restored in favour of $NO^{\bullet}$, the result could be inhibition of LDL oxidation by reaction with peroxyl radicals [13,31]. How effective is $NO^{\bullet}$ as a peroxyl radical scavenger? We can compare it with α-tocopherol which scavenges peroxyl radicals about 10,000 times faster than peroxyl radicals can recruit unmolested fatty acids into the cycle of lipid peroxidation. Although the concentration of α-tocopherol (30 μM in plasma) is approximately 100 times higher than the average concentration of $NO^{\bullet}$ in the artery wall (approx. 0.1–0.4 μM) $NO^{\bullet}$ reacts with peroxyl radicals with a rate constant of approximately $1 \times 10^9\ M^{-1} \cdot s^{-1}$ which is 10^3 times higher than the scavenging of these radicals by α-tocopherol. This simple and rather conservative calculation suggests that $NO^{\bullet}$ is a more effective antioxidant than vitamin E. However, the biological properties of the lipid-peroxyl-nitric oxide adducts (LOONO; Fig. 1) remain to be characterised before we can assess the overall consequences of this reaction.

3.3. Inflammation

ROS and RNS play important roles in killing foreign organisms and in acute inflammation but their over-production may cause tissue damage and vascular leakage in septicaemia, rheumatoid arthritis and inflammatory bowel disease [2,6,30]. The precise mechanisms of these cytotoxic effects are unknown but RNS are implicated since nitrotyrosine (Fig. 2) has been detected in serum and synovial fluid from rheumatoid patients [32] and co-localises with NOS in a guinea pig model of chronic gut inflammation [33]. Furthermore, intra-colonic instillation of peroxynitrite can produce colitis in rats [34] and patients with active ulcerative colitis show a marked increase in NOS activity in the inflamed colonic mucosa [35]. Septicaemia is associated with the induction of NOS in several cell types in response to mediators such as cytokines [6]. The iNOS isoenzymes are capable of sustaining high rates of $NO^{\bullet}$ production (see Fig. 1). In addition to the well recognised effects of $NO^{\bullet}$ in causing hypotension in this syndrome, vascular damage may also arise from RNS [2]. Acute respiratory distress syndrome (ARDS) which often results from severe infection, may also involve RNS generation in the lung as evidenced by the presence of nitrotyrosine [36].

3.4. RNS and cancer

Although RNS may be used by phagocytes to kill cancer cells, it is ironic that, like ROS, they have many of the characteristics of carcinogens [37,38]. Structural alterations in the DNA, e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification can be induced by ROS ($OH^{\bullet}$, $^1O_2$, $RO_2^{\bullet}$, $RO^{\bullet}$, O_3) and several RNS (HNO_2, $ONOO^-$) [1,37,39]. Nitrous acid deaminates DNA bases whereas $ONOO^-$ and products derived from it may well act as deaminating, nitrating and nitrosating agents, forming such species as 8-nitroguanine. RNS could cause alterations in cell signalling and may alter cytoplasmic and/or nuclear signal transduction pathways, e.g. nitration of tyrosine (Fig. 2) may prevent the action of tyrosine phosphatases and kinases. $NO^{\bullet}$, perhaps formed by $NO^{\bullet}$ synthases produced in the tumour could facilitate tumour growth by promoting angiogenesis. Indeed increased induction and activity of NOS in human gynecological cancers is correlated with increased malignancy [40].

3.5. Neurodegenerative disease

High levels of $NO^{\bullet}$ have been implicated in the pathology of Alzheimers disease, the consequences of viral infections of the CNS and in the excitotoxic neuronal death that is known to play a role in stroke and several neurodegenerative diseases [41–43]. Activation of the glutamate receptors triggers calcium influx into the cell, which stimulates the activity of calcium/calmodulin-dependent neuronal NOS under conditions where ROS formation is also enhanced. It follows that ROS/RNS interactions may also be important in these effects on the CNS. Microglial cells are essentially macrophages resident in the

brain and might be expected, on activation, to generate RNS as well as ROS. It is possible that reversible inhibition of cytochrome *c* oxidase by NO• leads to increased O_2^{-}• formation in mitochondria, $ONOO^-$ formation and resultant damage to FeS centres [7,8,11]. This idea is consistent with a role for both RNS and ROS in the impairment of mitochondrial function known to be associated with Parkinson's disease and possibly important in Alzheimers.

A connection between the neurological disorder amyotrophic lateral sclerosis (ALS), $ONOO^-$ and SOD has been suggested [44,45]. Patients with the familial dominant form of this disease (about 10% of the total patients) have mutations in Cu/Zn-SOD which could decrease enzyme activity by 40–60%. One hypothesis is that mutated SODs might promote damage to motor neurone cells by $ONOO^-$ dependent nitration of proteins [45].

4. Prospects for therapy: striking a balance

From the arguments outlined above it is evident that therapeutic intervention is confounded by not knowing at the outset whether the optimal strategy is to enhance or inhibit NO• formation. Clearly, a sound understanding of the pathological process is needed prior to intervention, perhaps utilising markers of RNS dependent damage outlined in Fig. 2. Below we outline some of the approaches which are either currently available or in development.

4.1. Nitric oxide synthase inhibitors

Under conditions where excess generation of NO• is contributing to the tissue damage, inhibition of NOS would seem to be a reasonable therapeutic strategy. However, given the widespread function of NO• in the body it would probably be advantageous if selective inhibition of the specific isozyme of NOS which is contributing to the disease process can be achieved [46]. Considerable progress has been made in the development of such compounds. For example, potent selective inhibitors of iNOS such as *N*-iminoethyl-L-lysine have been shown to suppress adjuvent induced arthritis in rats [47] and similar compounds are being considered for treatment of life threatening hypotension in septicaemia [2]. Similarly, selective nNOS inhibitors may be valuable in treating stroke. Other less selective inhibitors may be useful in an intensive care setting, where potential side effects such as hypertension can be carefully monitored.

4.2. Nitric oxide donors

Nitrovasodilators such as glycerol trinitrate have been known for years to be metabolised by vascular smooth muscle cells to release NO•. They provide immediate relief from the pain of angina by relaxing the constricted coronary vasculature. Their effects are short term because they induce tolerance probably through a O_2^{-}•-dependent mechanism [48]. New generations of NO• donors may allow selective release of NO• in different tissues and local restoration of the balance between RNS and ROS. For example, nitrosoglutathione appears to show platelet selective effects when administered in vivo whereas the classical organic nitrates have little or no effect on platelets at therapeutic doses [49]. The clinical indications of such compounds may then be much broader than angina and could include prevention of the re-occlusion of coronary arteries after angioplasty [50] and arresting premature labour [51]. Interestingly NO• itself is being used in the treatment of pulmonary hypertension [52].

Arginine administration may also have beneficial effects by enhancing NO• formation in the vasculature [24]. Several dietary antioxidants can inhibit $ONOO^-$-dependent damage; they include ascorbate and vitamin E [19]. Our knowledge of dietary effects on RNS in vivo is clearly in its infancy.

4.3. Scavenging of O_2^{-}•

One obvious way to restore the balance between NO• and O_2^{-}• would be to use SOD or SOD mimetics. However, because of the rapid reaction between NO• and O_2^{-}• localisation of the enzyme or compound to the vascular endothelium will be necessary. One approach is to use recombinant EC-SOD itself or genetically engineered SOD containing a heparin binding site [53].

5. RNS: the future

In this minireview we have focussed on the emerging ideas of how the balance between RNS and ROS may play a role in human disease, the insight and opportunities this provides for applying and understanding established therapies, and the approaches this may suggest for developing novel drug candidates. An area we have not addressed in any detail, yet is probably destined to take centre stage, is the effects of the changing RNS/ROS balance on the molecular events which control cell signalling and gene expression. From these studies it may emerge that under non-pathological conditions the RNS/ROS balance may play an important role in controlling cell growth, motility and life span.

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