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2012 J. Phys. D: Appl. Phys. 45 263001

(http://iopscience.iop.org/0022-3727/45/26/263001)

IOP PUBLISHING JOURNAL OF PHYSICS D: APPLIED PHYSICS

J. Phys. D: Appl. Phys. **45** (2012) 263001 (42pp) doi:10.1088/0022-3727/45/26/263001

TOPICAL REVIEW

The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology

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Received 6 January 2012, in final form 14 April 2012
Published 13 June 2012
Online at stacks.iop.org/JPhysD/45/263001

Abstract

Reactive oxygen species (ROS) and the closely related reactive nitrogen species (RNS) are often generated in applications of atmospheric pressure plasmas intended for biomedical purposes. These species are also central players in what is sometimes referred to as 'redox' or oxidation–reduction biology. Oxidation–reduction biochemistry is fundamental to all of aerobic biology. ROS and RNS are perhaps best known as disease-associated agents, implicated in diabetes, cancer, heart and lung disease, autoimmune disease and a host of other maladies including ageing and various infectious diseases. These species are also known to play active roles in the immune systems of both animals and plants and are key signalling molecules, among many other important roles. Indeed, the latest research has shown that ROS/RNS play a much more complex and nuanced role in health and ageing than previously thought. Some of the most potentially profound therapeutic roles played by ROS and RNS in various medical interventions have emerged only in the last several years. Recent research suggests that ROS/RNS are significant and perhaps even central actors in the actions of antimicrobial and anti-parasite drugs, cancer therapies, wound healing therapies and therapies involving the cardiovascular system. Understanding the ways ROS/RNS act in established therapies may help guide future efforts in exploiting novel plasma medical therapies. The importance of ROS and RNS to plant biology has been relatively little appreciated in the plasma biomedicine community, but these species are just as important in plants. It appears that there are opportunities for useful applications of plasmas in this area as well.

(Some figures may appear in colour only in the online journal)

1. Introduction

Study of the possible therapeutic uses of ionized gas plasmas is experiencing rapid growth by researchers worldwide [1–5]. However, the plasma-induced chemical and physical effects that are responsible for observed treatments are often challenging to interpret. The first challenge is that the plasmas themselves are difficult to fully characterize and model. Furthermore, their biologically active targets are also extremely complex with feedback loops and nonlinear couplings, and with invariably many unmeasured and no doubt even unknown variables playing important roles. The latter topic is part of the realm of 'systems biology' and is in itself an enormous subject. The field of plasma biomedicine is in need of some principles to serve as hypotheses to guide future scientific and clinical explorations. There is no doubt that

plasmas used for medical therapy generally create copious quantities of reactive oxygen and nitrogen species (ROS/RNS or RONS) [1]. It is equally clear that these species are important in biology and medicine [6]. However, it is less well known that in some cases, both recent and not-so-recent research demonstrates that RONS and redox biology in general are central to some important existing and emerging therapies. This general association helps provide a plausible hypothesis for the reports of successful applications of plasma to various maladies, but the question of how plasma-generated RONS delivered to cells and tissues interact in the 'biological milieu' remains largely unanswered.

The purpose of this non-exhaustive review is to identify some of these aspects concerning the actions of RONS, and to suggest that plasma biomedicine researchers consider these species as the probable active agents in related biomedical observations. The scope of the literature on RONS in biology and medicine is truly vast, rapidly growing, and is clearly well beyond any single review, or even monograph or text for that matter, so of course much will be left out. This paper attempts to identify some of the more useful (i.e. most relevant to plasma biomedicine) published works in the field, but it is inevitably incomplete and the reader is encouraged to use the present effort as a starting point only. This is also true for plasma biomedicine results themselves; this paper makes no attempt to review this field as there are multiple recent reviews that have addressed this topic [1, 2, 4, 7, 8].

The importance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in biology is difficult to exaggerate. In a comprehensive recent review, Novo and Parola [9] write:

> At present, redox research is at the forefront of biomedical research in view of the expanding knowledge on the roles that increased and/or sustained levels of oxidative stress and related mediators have been described to play in major human diseases, including atherosclerosis, diabetes and cardiovascular diseases, cancer, neurodegenerative disorders, chronic liver and lung diseases, to name just a few.

The potentially damaging effects of radicals have been known or suspected for decades, but it has become clearer more recently that the situation is not simply that radicals are uniformly damaging and any intervention that reduces their concentration is therapeutic. For example, in another review, (focusing mainly on plant biology, a topic considered in greater detail below), Halliwell [10] writes:

> In fact, antioxidants/free radicals permeate the whole of life, creating the field of redox biology. Free radicals are not all bad, nor antioxidants all good. Life is a balance between the two: Antioxidants serve to keep down the levels of free radicals, permitting them to perform useful biological functions without too much damage.

The 'two-edged sword' nature of RONS in biology in general and medical therapies in particular is a constant refrain in the literature cited in this paper. Over the past several decades, the pathogenicity of RONS has been emphasized. A well-known example is the 'free radical theory of ageing,' which posits that most of the processes that contribute to degenerative ageing processes are due to excess concentrations of radicals [11, 12]. However, based on exhaustive studies over the last several decades, most researchers now agree that although radicals are not the *cause* of ageing, radicals are undoubtedly *associated* with ageing and age-related disease [13]. Foyer and Noctor [14] note in this context that:

> The field of redox biology has recently witnessed a dramatic reappraisal of the function of reactive oxygen species and antioxidants. For many years considered as 'molecular hoodlums' to be suppressed or policed by the antioxidant system, ROS, like the low molecular weight antioxidants, are now considered to be dynamic information-rich signaling molecules.

These authors make the interesting point that antioxidants, as reaction partners for ROS, are themselves signalling molecules. With respect to the effects of ROS on organism lifetime, there is some evidence now that ROS, acting through mitochondrial metabolism, are sometimes associated with *increased* lifespan, as described by Ristow and Zarse [15], Ristoe and Schmeisser [16] and Hekimi *et al* [17].

It is only relatively recently that the *therapeutic role of reactive species* has been investigated in some detail, and this is one main focus of this paper. In essence, plasma biomedicine seeks to controllably and therapeutically administer RONS exogenously (i.e. originating outside the body), and this has some antecedents. For example, cancer cells can be killed with reactive species created by gamma- and x-radiation therapy (thought to be predominately OH), photodynamic therapy (PDT) (thought to be predominately $^1O_2$) and some cancer chemotherapies (generating RONS that are not generally known). Since very similar or identical reactive species are created by plasmas, the recent demonstrations that plasma can shrink tumours *in vivo* or kill cancer cells *in vitro* may well be related to the earlier therapies. Recent developments in the use of redox active secondary metabolites (mostly, but not exclusively from plants) as drugs for cancer and autoinflammatory disease also seem related to the use of plasma-generated RONS for medical purposes [18].

It appears inescapable that the successful development of plasma biomedicine applications will hinge in significant measure on controlling the actions of the RONS created in the plasma by generating only the species that are needed and delivering them to the right place at the right time in the right concentration. Further, the methods and procedures used to deliver RONS from the plasma to the body will also be crucially important since by definition 'reactive species' will react relatively quickly when exposed to cells, tissues and biological fluids containing proteins, lipids and carbohydrates.

Nathan and Ding point out that exogenous sources of RONS include ultraviolet photons that generate $^1O_2$ and that also induce nitric oxide synthase 2 in skin, as well as smoke and air pollutants that contain ROS and organic radicals [19]. Not all exogenously delivered RONS are desirable,

Table 1. List of various reactive oxygen, nitrogen, halogen and sulfur species [10, 22, 23].

Radical	Non-radical	Radical	Non-radical
Reactive oxygen species (ROS)		*Reactive nitrogen species (RNS)*	
Superoxide, O_2^-	H_2O_2	Nitric oxide, NO	Nitrous acid, HNO_2
Hydroxyl, OH	Ozone, O_3	Nitrogen dioxide, NO_2	Nitrosyl cation, NO^+
Hydroperoxyl, HO_2	Singlet oxygen (O_2 1 Dg)	Nitrate radical, NO_3	Nitroxyl anion, NO^-
Carbonate, CO_3^-	Hypobromous acid, HOBr		Dinitrogen trioxide, N_2O_3
Peroxyl, RO_2	Hypochlorous acid, HOCl		Dinitrogen tetroxide, N_2O_4
Alkoxyl, RO			Dinitrogen pentoxide, N_2O_5
Carbon dioxide radical CO_2^-	Hypoiodous acid, HOI		Alkyl peroxynitrites, ROONO
Singlet ($^1O_2$)	Organic peroxides, ROOH		Alkyl peroxynitrates, RO_2ONO
	Peroxynitrite, ONOO−		Nitryl chloride, NO_2Cl
	Peroxynitrate, O_2NOO-		Peroxyacetyl nitrate, $CH_3C(O)OONO_2$
	Peroxynitrous acid, ONOOH		
	Peroxomonocarbonate, $HOOCO_2^-$		
	Carbon monoxide, CO		
Reactive chlorine/bromine species		*Reactive sulfur species*	
Atomic chlorine, Cl	Chloramines	Thiyl radical S.	Hydrogen sulfide, H_2S
Atomic Bromine, Br	Chlorine gas, Cl_2		Disulfide, RSSR
	Bromine gas, Br_2		Disulfide-S-monoxide, RS(O)SR
	Bromine chloride, BrCl		Disulfide-S-dixide, RS(O)2SR
	Chlorine dioxide, ClO_2		Sulfenic acid, RSOH
			Thiol/sulfide, RSR′

clearly. Understanding the balance between the pathogenic and therapeutic sides of RONS is certainly one of the keys to the future prospects of the technology. Finding the right balance raises questions familiar in toxicology about the nature of dose–response relationships. This is related to the concept of *hormesis*, or the tendency of compounds to be benign or to act therapeutically at low concentrations but become toxic at higher concentrations [20].

What species are generally included in this 'RONS' category? (Note that some authors use the terms reactive oxygen and nitrogen *intermediates* (ROI/RNI) rather than reactive oxygen or nitrogen *species*.) Nathan [21] points out that adding one electron to O_2 yields O_2^- (superoxide anion), adding two electrons creates H_2O_2, three electrons results in hydroxyl radical (OH) and a fourth electron yields water (H_2O). The idea of adding or removing electrons for chemical reactions to occur is characteristic of the class of oxidation–reduction ('redox') reactions: an oxidizing species is one that *removes* an electron from a reaction partner; the reducing species *accepts* an electron from the reaction partner. Note that ROS 'oxidation state' is not directly coupled with chemical reactivity since OH is much more reactive than the other species. A similar progression can be described for reactive nitrogen: nitric oxide (NO), nitrogen dioxide radical (NO_2), nitrite (NO_2^-), nitrate (NO_3^-) and peroxynitrite ($OONO^-$). Nathan includes ozone (O_3) and singlet O_2 ($^1O_2$) as ROS as well as hypochlorous (HOCl), hypobromous (HOBr) and hypoiodus (HOI) acids. A more complete but not exhaustive list, including reactive sulfur compounds, is provided in table 1 [10, 22–24].

Riley [25] observes that for the last 2 billion years, oxygen has served as the major electron sink in biological systems. ROS are endogenously produced during aerobic metabolism and via various enzymatic and some non-enzymatic reactions. These species participate in many important cell-signalling cycles and are centrally active in the immune systems of all multicellular organisms. Figure 1 illustrates some enzymatic and non-enzymatic pathways that have been identified through which various RONS are created in macrophages and neutrophils of the innate immune system [26]. Although not shown in figure 1, the various and unusually important roles played by the family of NADPH oxidase enzymes (creating O_2^-) have been reviewed recently [27, 28].

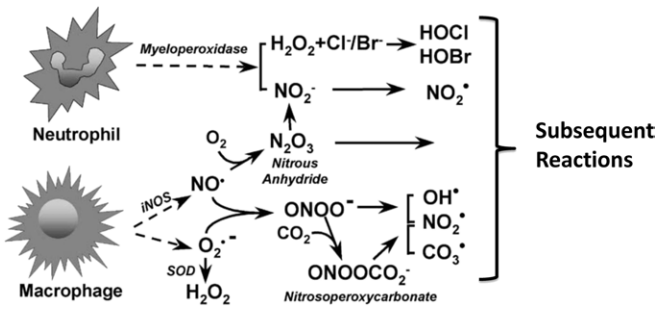

Figure 1. Following Dedon and Tannenbaum [26]. Reactive oxygen and nitrogen species created during inflammation from phagocytes, but not showing the effects on host DNA or other subsequent reactions.

The following sections of the paper begin with a brief summary of the major types of plasma sources that have been developed and applied for plasma biomedical applications. Model predictions of RONS from some of these sources are included (with apologies for the exclusive use of results from the author's laboratory). Because RONS play such an important set of roles in normal cell function, this very large subject is introduced in the following section, including the connection between RONS and mammalian innate and acquired immune systems. Although RONS are no longer necessarily identified as the primary causative agent in ageing and disease, they are certainly associated with a vast array of human maladies. This is summarized in the subsequent section, ending with a short description of recent ideas that

emphasize the positive roles of RONS in ageing, at least for laboratory studies of worms and rodents.

In the next section, several medical areas are highlighted in which RONS have been suggested or have been shown to be key players in established medical therapies. Briefly, this paper summarizes recent research showing that ROS are thought to be the key agents in all classes of antibiotics and for certain anti-fungal and anti-parasite drugs; that RONS are at the heart of many cancer therapies, including radiation therapy, PDT and even some chemotherapies; and that RONS play key roles in wound healing therapies. The penultimate section summarizes aspects of redox plant biology. RONS are known to be important in plant function and this connection hints at possible applications in horticulture and agriculture. The paper ends with concluding remarks, including reflections and speculations on future developments of plasma biomedicine.

The observations on the putative therapeutic roles of RONS in current, established therapies offer compelling hints that plasma-generated RONS are involved in present and future applications of plasma sources to healthcare. They also may suggest likely mechanisms of plasma biomedicine therapies by analogy. In addition, there is value for the acceptance of plasma medicine and healthcare in the wider medical research community if this nascent set of novel techniques can be plausibly associated with a set of well-developed and well-established therapies and associated scientific principles. Finally, it is hoped that these results will offer guides to researchers in plasma biomedicine on possible approaches, orientations, methodologies and potentially fruitful hypotheses for future investigations.

2. Atmospheric pressure plasma sources of RONS

2.1. Introduction

One of the important factors associated with the growing interest in plasma applications to biomedicine is the development of new or modified atmospheric pressure low-temperature plasma sources. By 'low temperature' is meant weakly ionized gas plasmas for which the neutral species are usually near-room temperature while electrons are hot—typically several electron volts in average energy. It should be noted that some sources operate with relatively high gas temperature in the plasma region, then use gas convection and heat conduction to reduce the temperature of plasma-generated active species before contacting tissue. Ions are usually near neutral temperature in the plasma region but may be accelerated to higher kinetic energies in sheaths, just as in low pressure non-equilibrium plasmas [29–31]. As is well known, the hot electrons generally sustain the plasma by direct electron-impact ionization and/or via the formation of various excited states that create ion–electron pairs. In addition to ionization, these hot electrons will dissociate molecular gases, including oxygen and nitrogen. Photons from the vacuum ultraviolet to infrared are created; electric fields drive currents passing through the gas and onto bounding surfaces and charges may accumulate on those surfaces. In principle, any or all of these effects could be important biomedically. However, it is the neutral–neutral and ion–molecule chemistry that results in the creation of RONS as well as a suite of other reactive chemical species, both radical and non-radical, which are the focus here. The primary purpose of this section is to point out some of the evidence, both experimental and modelling, that such sources are copious and flexible producers of RONS and other reactive species.

Sometimes the discharges are sustained in spatially confined regions and are called 'microplasmas.' [32] Although the plasmas of interest for plasma biomedicine are not necessarily strictly microplasmas or even arrays of microplasmas, there are usually significant similarities. Iza *et al* [31] provide an overview of microplasmas with an emphasis on biomedical applications.

These plasma sources can be relatively inexpensive, with convenient and healthcare friendly designs, possibly handheld and portable and, as noted above, are capable of creating chemically active species near-room temperature. Some designs lend themselves to endoscopic operation. Discharges creating low-temperature plasmas at atmospheric pressure are usually not completely homogeneous spatially and may be transient (pulsed or simply fluctuating randomly) as well.

There have been multiple recent reviews describing the effects of various plasma sources on biomedical targets. (e.g. [1–3, 33]) Applications include antimicrobial sterilization/decontamination/antisepsis, blood coagulation, wound healing and other aspects of dermatology, anti-tumour effects, dental applications and various effects on cells *in vitro* (e.g. [4, 5, 8]). In the discussion that follows, some recent biomedical applications will be described that are associated with particular plasma sources, but the presentation is not exhaustive and many other analogous devices have been developed around the world.

2.2. Plasma torches

A 'plasma torch' in this context is a discharge in which the gas in the vicinity of the plasma itself is relatively hot, but gas convection through this region carries neutral species downstream and cooling is sufficient that the gas is not too hot to contact living tissue. One such design has been discussed and used by Morfill and colleagues for a variety of biomedical applications [3]. In this system (see figure 2) a flow of several standard liters per minute (slm) Ar passes through a 2.45 GHz microwave-powered torch and flows downstream to the surface to be treated. These authors have characterized the source, including gas temperature, ultraviolet photon (uv) flux and some of the neutral chemistry. For example, they measured several ppm of NO_2 near the exit of the torch. Using one version of this device, Isbary *et al* [34, 35] report remarkable success in reducing bacterial loads in chronic wounds in one clinical study and in healing Hailey–Hailey disease, a skin disorder, in another study involving a single patient. In the latter study, these authors suggest that plasma treatment may contribute to healing in part through the bactericidal and fungicidal effect of the plasma species but also through affecting the oxidant–antioxidant system in the skin lesions.

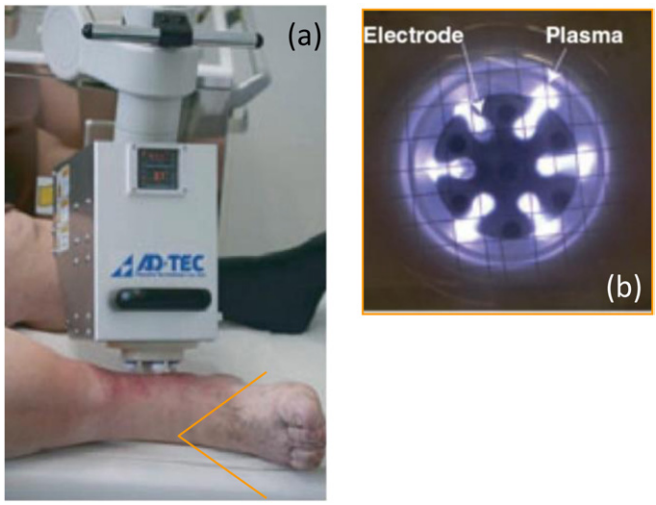

Figure 2. (*a*) Photograph of microwave plasma torch system applied to patient with chronic wound and (*b*) view of torch with electrodes and plasma through which argon flows [5, 34].

2.3. Rare gas jet discharges

In this category of plasma source, a flow of mostly either He or Ar through a dielectric tube is excited to form a jet of active plasma that may extend several centimetres past the tip of the tube. Other gases are often mixed into the rare gas, such as O_2 for example. The plasma is generally created by the application of radiofrequency voltages on one or two metal electrodes on the outside of the dielectric tube, as illustrated schematically in figure 3(*a*). They are thus 'dielectric-barrier discharges' (DBDs) in this sense, but are generally referred to as plasma jets because of the gas flow configuration. Figure 3(*b*) is a photograph of a typical device from the author's laboratory. One such configuration was named the 'plasma pencil,' a system designed by Laroussi [36]. Later studies showed that the plume emanating from the tip of the device, although it appears continuous to the eye, is in fact a series of very rapid light pulses. These are sometimes referred to as 'plasma bullets,' and have been the subject of much discussion and debate in the literature [37].

Robert *et al* [38] present an especially promising 'plasma gun' configuration that generates plasma inside narrow, flexible dielectric tubes that persist for distances on the order of a metre. Interest in configurations such as this design centres around the obvious need to deliver plasma endoscopically for many potential medical applications.

Other rare gas jet designs have been reported and characterized, for example, by Kong *et al* [1], Knake and Schulz-von der Gathen [39], Sousa *et al* [40] and Weltmann *et al* [33]. ROS measured in these sources include O atoms via two-photon absorption laser-induced fluorescence [39] and $^1O_2$ via infrared optical emission spectroscopy [40]. Ikawa *et al* [41] used a similar rare gas jet device to study the effects of RONS in water solution on bacterial suspensions. Benedikt and co-workers report results using a novel design that can separate the effects of neutral molecules and photons [42].

The so-called plasma needle (see figure 4(*a*)), originally introduced by Stoffels and co-workers, is also a rare gas plasma jet. A photo of one version of the plasma needle is shown in figure 4(*b*) [43]. In this design, a flow of He in a small diameter (∼1 cm diameter typically) past a thin needle electrode powered at 13.56 MHz, creates a plasma at the tip of the sharp needle that may extend to a surface several millimetres from the tip. Sometimes a small amount of another gas such as O_2 is included in this flow, creating various ROS. But even in the absence of added gases, RONS are generally created at the boundary between the rare gas jet and the adjacent air through various mechanisms. This design has been modelled [29, 30, 44, 45] and been the subject of several chemical diagnostic studies [46, 47]. Figure 4(*c*) reproduces some model predictions of various RONS crated in a plasma needle discharge, showing clear evidence of the reactive species that are the main topic of this paper [48].

2.4. DBDs—direct and indirect

DBDs, as illustrated in figures 5 and 6, can be operated 'indirectly' or 'directly,' respectively. In the indirect mode, the plasma remains near the powered electrode and is not in contact with the treated surface. Chemical species created in the plasma either diffuse or are transported via forced or natural convection to a treated surface (see figure 5(*a*)). Figure 5(*b*) is a photograph from the author's laboratory of a typical indirect DBD design, sometimes referred to as a surface microdischarge (SMD) device, in which a grounded mesh next to the dielectric layer effectively confines the plasma to a region several millimetres thick (see, e.g., [3, 49]). Figure 5(*c*) shows results from recent model predictions of air SMD discharges with either no water vapour or 30% humidity [48]. Finally, several groups have shown that air plasma created via indirect DBD, when in the vicinity of unbuffered water for minutes to tens of minutes, will form an acidic solution containing nitrous (HNO_2) and nitric (HNO_3) acids, as well as H_2O_2. It is generally acknowledged that the solutions contain other species as well, but more detailed water composition analysis has not been reported. The steady state pH is about 2.8. (e.g. [49, 50]) The significance of this water chemistry will be apparent in later sections of the paper when discussing formation of secondary products such as nitrated lipids in tissue and cells following air plasma exposure.

In direct mode, as illustrated schematically in figure 6(*a*), the plasma is in direct contact with the treated surface. The difference is that current then flows to the treated surface and charged particles (electrons and ions) will generally impact the surface, possibly changing the surface chemistry. Figure 6(*b*) is a photograph from the author's laboratory of a relatively small device; the electrode areas can be scaled to be larger than shown here. In general, direct DBD discharges generate transient, filamentary discharges, raising question sometimes about the uniformity of surface treatment.

3. Roles of RONS in normal cellular homeostasis

3.1. Introduction and history

Al Ghouleh *et al* [51] recount the history of discoveries associated with the role of ROS in the immune system. In

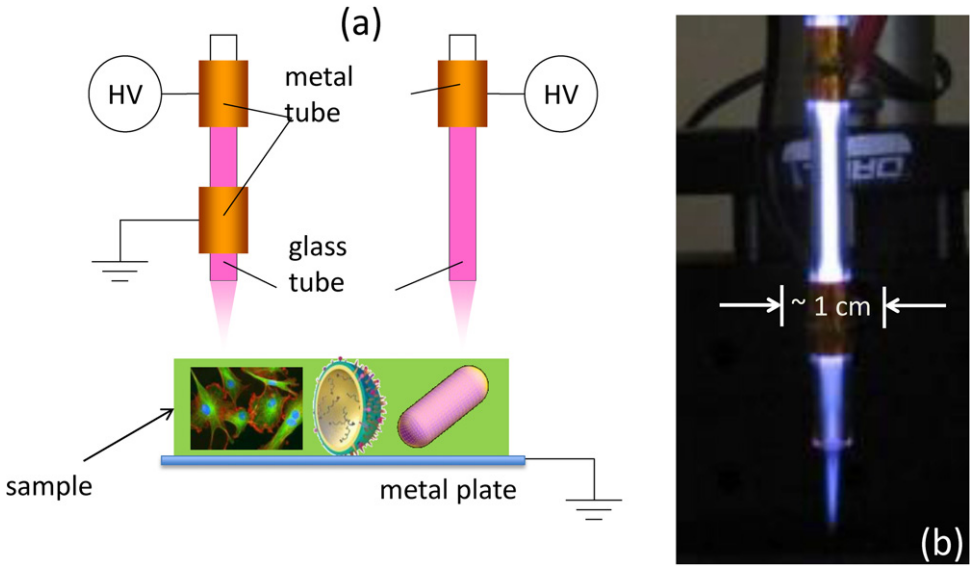

Figure 3. (*a*) Sketches of rare gas jet configurations with sample treated and (*b*) photograph of typical laboratory He jet device.

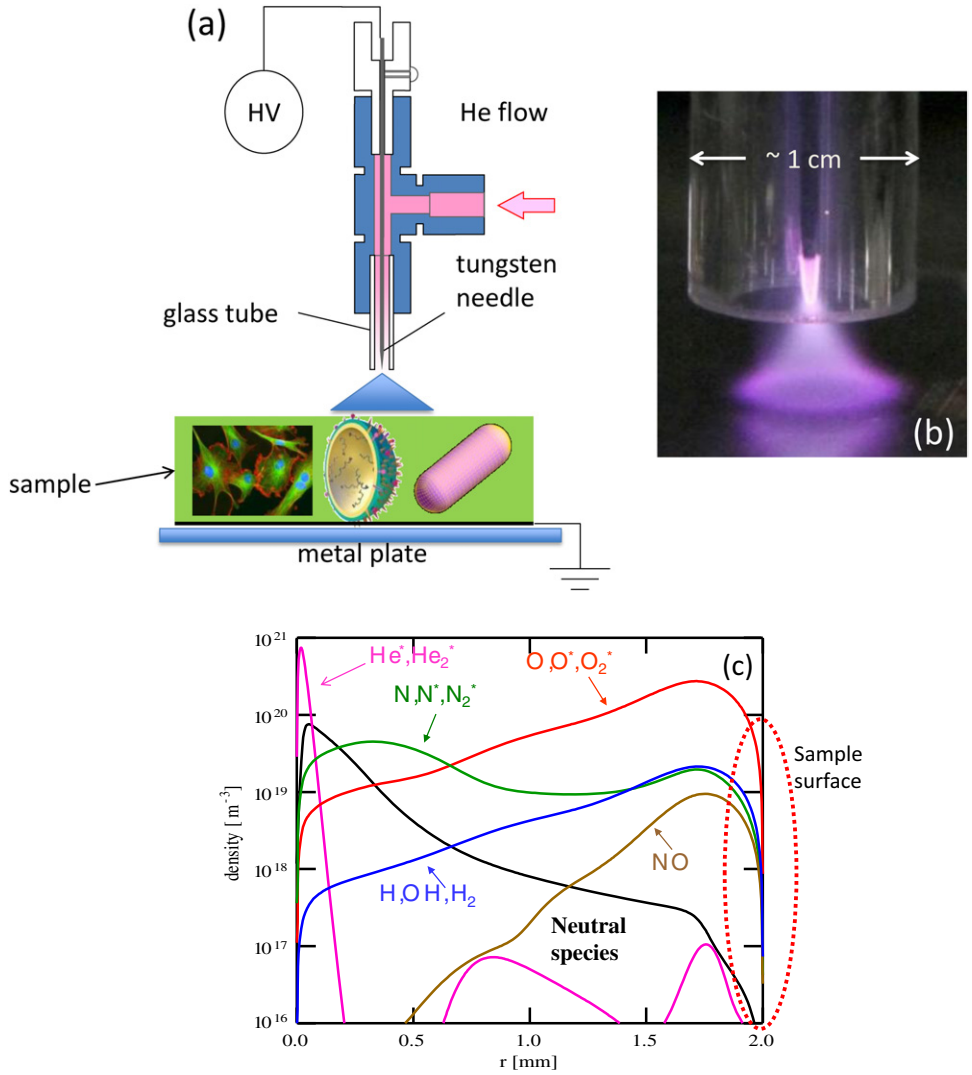

Figure 4. (*a*) Sketches of helium plasma needle configuration with sample treated, (*b*) photograph of typical laboratory device. (*c*) Plot of neutral reactive species densities, following plasma model of He plasma needle near plasma-air boundary, with sample boundary at $r = 2.0$ mm [48].

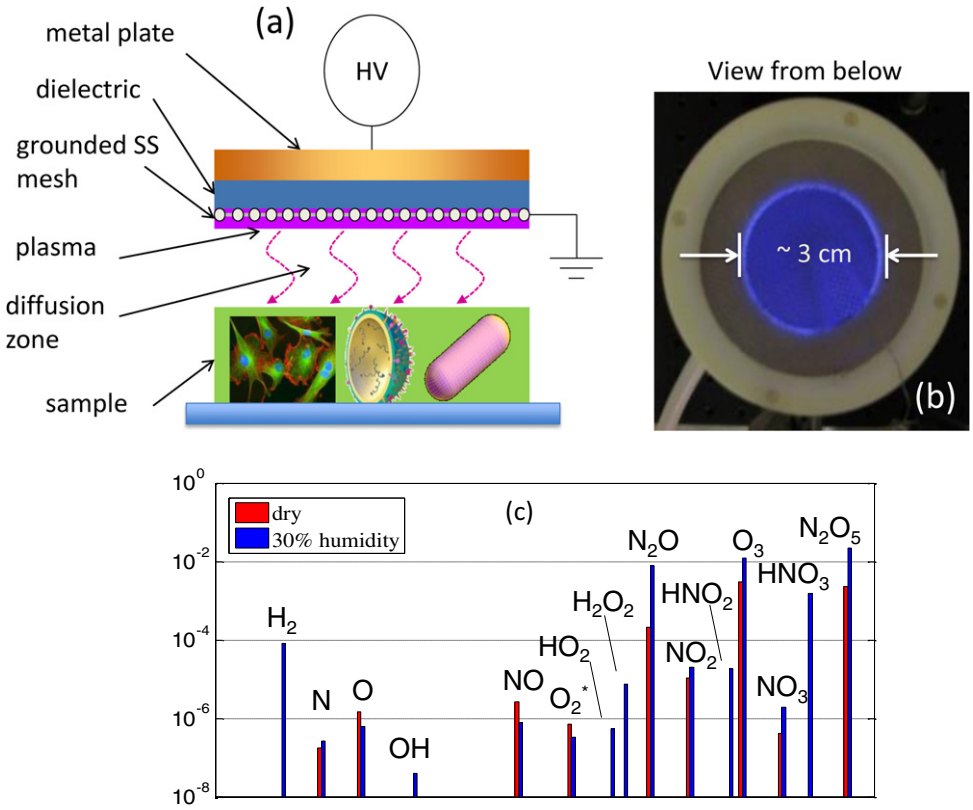

Figure 5. (*a*) Sketch of indirect DBD configuration with sample treated, (*b*) photograph of typical laboratory indirect DBD device operating in room air and (*c*) model prediction of dry and humid air plasma chemistry at processed surface [48].

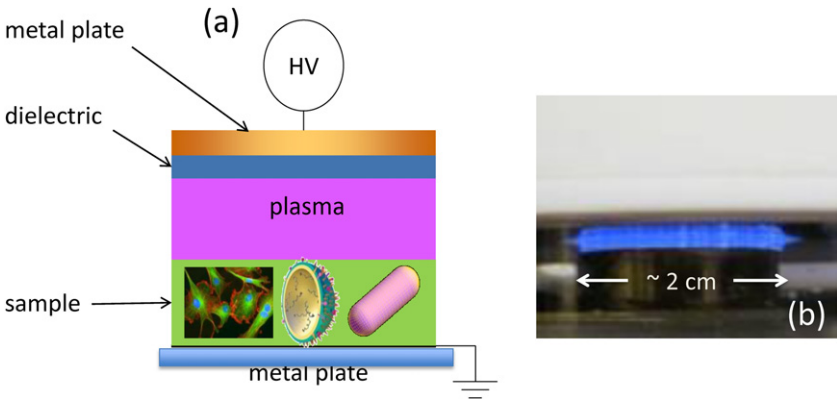

Figure 6. (*a*) Sketch of direct DBD configuration with sample treated and (*b*) photograph of typical laboratory DBD device operating in room air.

1932, the 'respiratory burst' from phagocytosing leukocytes was first reported [52], followed in 1961 by the discovery that the oxidizing species released is H_2O_2 [53], then in 1973 it was shown that this hydrogen peroxide is formed from O_2^-, a one electron reduction product of O_2 [54]. In 1954, Commoner, Townsend and Pake published what is generally regarded as the first paper measuring free radicals in biological (lyophilized or freeze-dried) tissue, with measurements of radicals using electron paramagnetic resonance (EPR) [55]. The same year, in an apparently independent investigation, Gerschman *et al* [56] were motivated by various reports of the roles of redox/radical chemistry in biology (e.g. [57]) to investigate the hypothesis that oxygen poisoning and radiation injury were at least in part related through the damaging effects of oxygen free radicals. Their studies led them to conclude that oxygen free radicals are indeed involved in both types of injury. Two years later, a very influential paper by Harman was published in which he noted that radicals created in cells exposed to radiation were very damaging to the exposed organism, leading to cancer, mutations and ageing [11]. By analogy, he reasoned that radicals in living systems, perhaps created in oxidative enzymatic reactions or from traces of unliganded transition metals, might also lead to ageing. This became known as the 'free radical theory of ageing.' The term was later modified to the 'mitochondrial free radical theory of ageing' because of the observation of especially ROS created apparently as inevitable but essentially inadvertent side effects in the mitochondrial electron transfer chain.

McCord and Fridovich in 1969 reported their discovery of an enzyme that acts to convert the superoxide anion (O_2^-) to hydrogen peroxide—the enzyme now referred to as 'superoxide dismutase' (SOD) [58]. The fact that organisms create this enzyme implied that radicals were somehow 'natural'. This observation convinced many biologists that oxidative free radicals were important in biology and it was worth studying their (assumed largely damaging) effects. It was soon recognized that there were multiple ROS and they could cause damage to proteins, lipids and carbohydrates [59]. The concept of 'oxidative stress' resulting from an imbalance between oxidizing and antioxidizing species began to be widely adopted in the mid-1980s. It was also recognized, as noted above, that these ROS were involved in a positive way in phagocytes in the innate immune system, but the major focus of studies of radicals and oxidative stress was on their disease-causing role. The dominant concept at this time, and this persists even to the present although it is now more widely acknowledged to be at best incomplete, is that (mostly damaging) radicals present in the body need to be minimized or countered with antioxidants. For the more enlightened current view, see, e.g., Gutteridge and Halliwell [60]. Indeed, recent research has shown that antioxidant supplements are, at best, no help in prolonging life [61].

Appreciation of the importance of reactive nitrogen in biology developed later than it did for reactive oxygen. It was not until the late 1980s that it was realized that the radical nitric oxide (NO) plays a variety of important biological roles, including in vascular relaxation, among others. Hibbs *et al* [62] established that macrophage antimicrobial effects depend on L-arginine to create compounds related to nitrite and nitrate (NO_2^-/NO_3^-). Marletta *et al* [63] showed that NO is an intermediate product in the formation of NO_2^-/NO_3^- from L-arginine in immuno-stimulated mouse macrophages, and they suggested that macrophage-generated NO plays a signalling role in analogy with its vasodilation role in endothelium tissue. Stuehr and Nathan [64] demonstrated that NO (or a closely related product) is the key active species in macrophage cytostatic action against microbial and tumour targets. Ischiropoulos *et al* [65] showed that peroxynitrite ($OONO^-$) is also created in macrophages. In 1998, Furchgott, Ignarro and Murad received the Nobel Prize in Medicine or Physiology 'for their discoveries concerning NO as a signalling molecule in the cardiovascular system.'

Pacher *et al* [66] recount the history of nitric oxide discoveries in biology in greater detail. A series of studies showed that reactive oxygen and reactive nitrogen species (RONS) were involved in key signalling processes within the cell. In 'redox' (or oxidation–reduction) signalling, information that arrives at the membrane of the cell (the 'first messenger') via ligand–receptor binding can be transferred throughout the cell (the 'second messenger') through a series of electron transfer reactions involving radical and non-radical reactive species. As discussed below, the details on how this works with RONS are still emerging.

de Castro Fernandes *et al* [59] make the point that redox processes are important in biology and medicine in part because 'ancestral and ubiquitous' redox reactions pervade all of aerobic life and play important roles in homeostasis (maintenance of stable conditions) in all prokaryotic (bacterial) and eukaryotic (plant/animal) cells. Further, redox reactions 'pack a punch'—these reactions are among the most powerful that can be marshaled in biological systems, and are among the few reactions with sufficiently large free energy changes necessary to supply the heavy metabolic demands associated with higher living organisms [67].

The emerging view of redox biochemistry and the role played by the various reactive species involved is that these species have been an intrinsic part of cellular biochemistry since the beginning of aerobic life. Their actions and characteristics are woven into virtually all inter- and intracellular signalling networks with a finely tuned chemical balance between different parts of the complex system. The notion that one set of species in this network is 'bad' and another 'good' is clearly naïve. Nevertheless, even though RONS are key species when properly controlled within the aforementioned homeostatic networks, there seems to be little doubt that harmfully high concentrations of RONS, or RONS-generated products are associated with a vast range of disease states and are also associated with ageing, and this topic is summarized in a later section of the paper.

3.2. Introduction to cellular redox signaling mechanisms

As noted above, many important details of cell signalling via RONS and redox reactions are still a subject of very active research, and are certainly still being widely debated in the literature. A key idea for RONS in cell-signalling networks is that these relatively small species tend to form covalent bonds. This is in contrast with traditional ideas of signalling dynamics in which two macromolecules engage in a kind of non-covalent 'handshake' as one folds around or fits into another in a key-lock configuration. This local macromolecular interaction can be mediated by shape, charge, hydrophobicity and so forth, and may lead to covalent bond formation or rupture such as adding or removing a phosphate group (PO_3^-), termed phosphorylation or de-phosphorylation. This change in protein shape leads in turn to other reactions, and can ultimately result, for example, in transcription and translation to form new proteins or many other cellular outcomes. But what are some ways that RONS are thought to participate in signalling cascades? The introductory discussion provided below is neither exhaustive nor necessarily based on fully settled science but is intended as an introduction to some of the topics and issues being debated and studied.

Hydrogen peroxide (H_2O_2), at least in the absence of nitric oxide (discussed further below) is thought to be one of the most important signalling molecules among the RONS [68]. For example, figure 7 (following Rhee [69]) illustrates one view of the role of intracellular H_2O_2 signalling. In this figure, the O_2^- creating enzyme 'Nox' is activated when a ligand binds to a receptor at the cell membrane. Nox can be in the membrane or internal to the cell in an organelle. The O_2^- created by Nox is converted to H_2O_2 (typically via SOD), and is either imported into the cell if made outside the cell, or is made in the organelle and moves to the cytosol (the intracellular

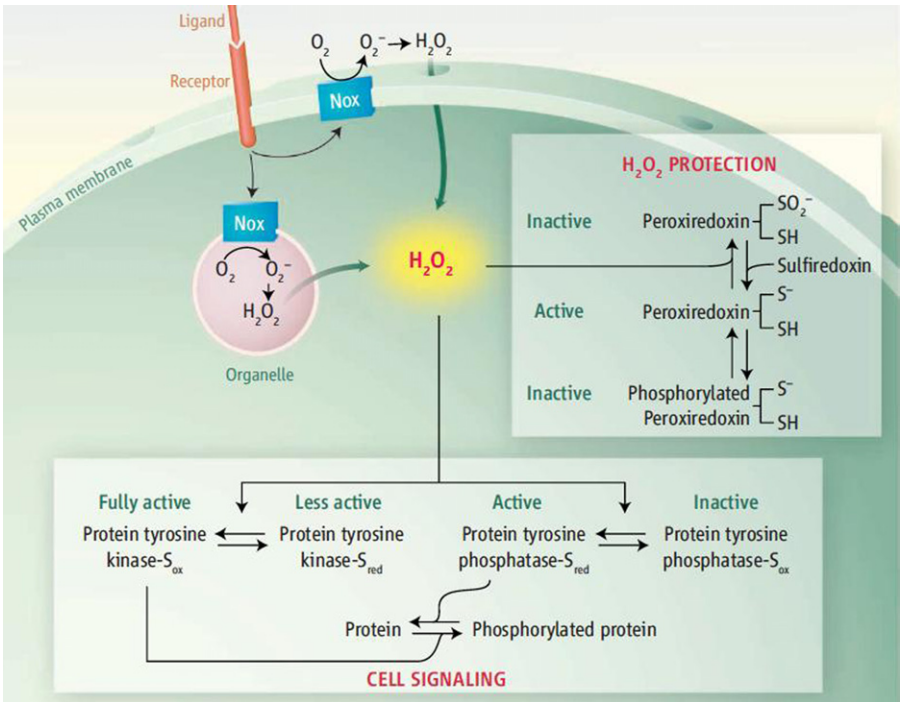

Figure 7. Following Rhee [69]. Receptor–ligand binding at the cell membrane activates the Nox (i.e. NADPH oxidase) enzyme either in the plasma membrane or in an internal structure such as an endosome, forming H_2O_2. Once inside the cytosolic intracellular fluid, H_2O_2 inactivates protein tyrosine phosphatases while activating protein tyrosine kinases. H_2O_2 is also thought to overwhelm the cytosolic reducing agent peroxiredoxin by either hyperoxidation or phosphorylation.

fluid). Once in the cytosol, H_2O_2 reacts with (i.e. oxidizes) two enzymes, both in the reduced state, to transform them into their oxidized states. One target is protein tyrosine kinase. Like all enzyme kinases, this enzyme acts to phosphorylate proteins containing tyrosine. The reaction with H_2O_2 *activates* protein tyrosine kinase in this proposed scheme. Additionally, H_2O_2 reacts with the reduced (active) form of protein tyrosine phosphatase, the enzyme that removes the phosphate group from proteins containing tyrosine. By reacting in this way, the proposed scheme shows H_2O_2 *inactivating* this enzyme. Thus, reaction with H_2O_2 promotes the phosphorylation of proteins with tyrosine by reacting covalently with both their creating and destroying enzymes. In addition, the figure shows that peroxiredoxin, a key reducing molecule that tends to remove H_2O_2 from the cytosol, is itself eliminated or at least diminished by H_2O_2 through either hyperoxidation (top path) or phosphorylation (bottom path).

Of course, H_2O_2, while clearly important, is not the entire story in RONS signalling mechanisms. The addition of RNS, especially nitric oxide (NO), changes the biochemistry dramatically. Figure 8(*a*) (following [67]) illustrates some of the major pathways associated with reaction between O_2^- and NO, showing the role of participating species such as O_2, CO_2 or Fe^{2+}, and the differences in these pathways that can result when one concentration is higher than the other. The main product between O_2^- and NO is the very important species peroxynitrite anion (ONOO-). If either O_2^- or NO is present in slight excess, the reaction leads to the nitrogen dioxide radical NO_2. Higher NO concentration leads to N_2O_3 and this often results in nitrosation (the addition of a NO moiety; addition of NO_2 is referred to as 'nitration'). If the concentrations of both NO and O_2 are relatively high, NO autoxidation forms NO_2. In the case of O_2^- present in greater concentration than NO, the result is the formation of peroxinitrate (O_2NOO-) or nitrite (NO_2^-). This condition can also lead to so-called 'Fenton' chemistry, in which OH is formed from the catalytic reaction of H_2O_2 with Fe^{2+}. This reaction scheme is illustrated in figure 8(*b*), starting with the formation of O_2^-, its conversion to H_2O_2 and the role of Haber–Weiss chemistry in reducing Fe^{3+} back to Fe^{2+}. OH radical is so reactive that it is thought to react with essentially any target. Finally, the presence of millimolar concentrations of CO_2 will lead to the formation of NO_2 from peroxynitrite.

One of the characteristics of reactive species is the fact that their relatively high reactivity limits the spatial extent to which they can diffuse once formed. One pictorial representation of this situation is provided by Pacher *et al* [66], and is illustrated in figure 9. O_2^-, NO, ONOO- and OH are all shown with their respective estimated diffusion distances associated with their 'half-life' (distance associated with a 50% reduction in concentration with respect to its concentration at its point of creation). The most reactive species, OH, will diffuse only a distance of about the diameter of a small protein before reacting, but peroxynitrite diffuses about 10 000 times farther. The length scales in the vascular region of the figure can be estimated with the 7 μm diameter of the red blood cell. Nitric oxide half-life is about 1s in this milieu and is controlled by uptake into red blood cells. These diffusion distances are obviously estimates based on assumed reactivity and concentrations of reacting partners, but they are fairly representative of estimates made by others. For example, Winterbourn and Hampton [70] provide a more

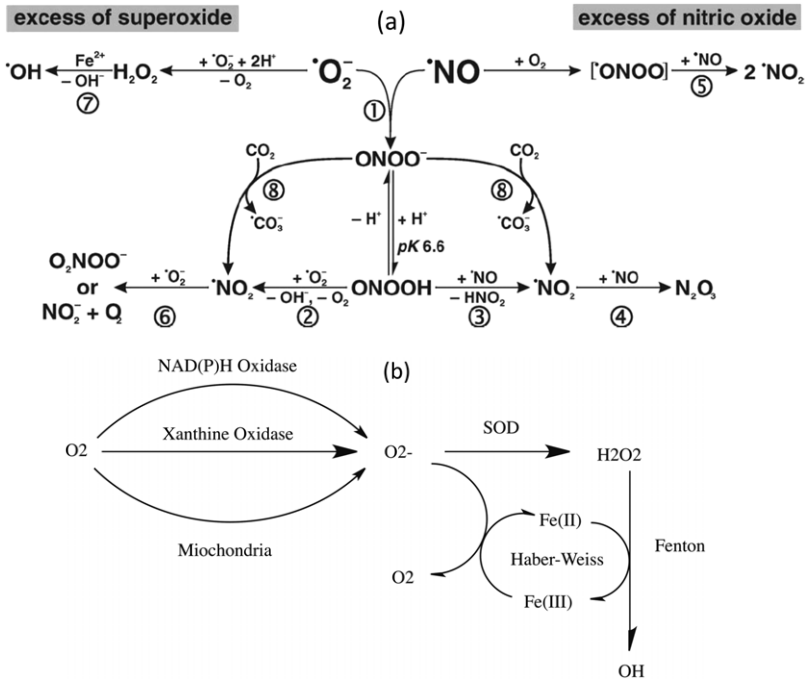

Figure 8. (*a*) Following Frein *et al* [67]. Reactions between superoxide and nitric oxide. (1) The main product between NO and O_2^- if present in equal amounts is peroxynitrite, OONO-. (2,3) Excess of either species shifts product towards nitrogen dioxide, NO_2. (4) Excess of NO leads to N_2O_3 and nitrosation (addition of NO). (5) Oxidation of NO by O_2 requires relatively high concentrations of both. (6) If O_2^- concentration exceeds NO, peroxynitrate (O_2NOO-) or nitrite (NO_2^-) form. (7) Excess O_2^- over NO leads to the Fenton reaction, forming the rapidly reacting radical OH, associated with oxidative stress. (8) High CO_2 concentration (∼1 mM) shifts decomposition of peroxynitrite towards forming NO_2. (*b*) Reactions forming O_2^-, H_2O_2 and OH. O_2^- is usually made either enzymatically from NADPH oxidase or xanthine oxidase or non-enzymatically from the electron transfer chain in mitochondria. O_2^- is converted to H_2O_2 by superoxide dismutase (SOD), or reduces Fe(III) to Fe(II) in the so-called Haber–Weiss reaction. H_2O_2 reacts with Fe(II) to form OH in the well-known Fenton reaction. This well-known chemistry is widely reported.

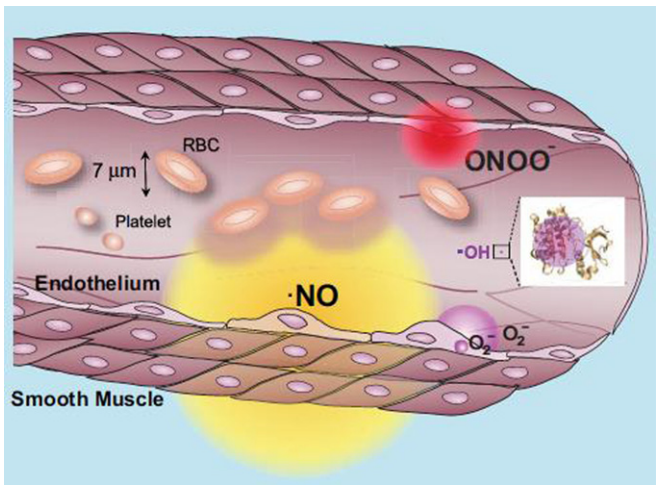

Figure 9. Following Pacher *et al* [66]. Estimated diffusion distances of NO, ONOO-, OH and O_2^- in a vascular context. Note the scale of the red blood cell of ∼7 μm. The diffusion distance of OH is expected to be only on the order of the size of a small protein. The NO 'half-life' of ∼1 s is estimated from its reaction with red blood cells. Pacher *et al* note the many approximations made for these estimates, but diffusion distances scale with the square root of characteristic reactions rates (or the inverse of the lifetime) so distances are less sensitive to uncertainties in reactivity.

detailed analysis and a wider range of chemistry on this topic. The question of RONS lifetime and diffusion distance is of crucial importance not only for cell-signalling mechanisms but also for any application of plasma-generated RONS for biomedical purposes.

3.2.1. Thiols in redox signalling. Two necessary characteristics for any proposed signalling mechanism are 'specificity' and 'reversibility.' Specificity refers to the fact that an information-transmitting signalling agent must have a limited but non-zero set of targets (i.e. it cannot react with all possible targets but it must react with something) and reversibility means that the signal can be turned off at some point. Both characteristics are easily and obviously associated with the kind of signalling mentioned above with macromolecules engaging in complementary folding, non-covalent interactions. But for RONS and their covalent bond-forming chemistry, how can this work? Selectivity in this case means that RONS can react with only a subset of possible cellular targets—either certain molecules or at least only certain functional groups. For example, OH cannot be a signalling mediator because it is much too reactive and will react with virtually anything, often creating such a strong covalent bond that it cannot be reversed enzymatically.

It is now well known that in order to satisfy these constraints, RONS signalling often involves macromolecules (typically proteins) containing so-called 'thiol' groups; a thiol

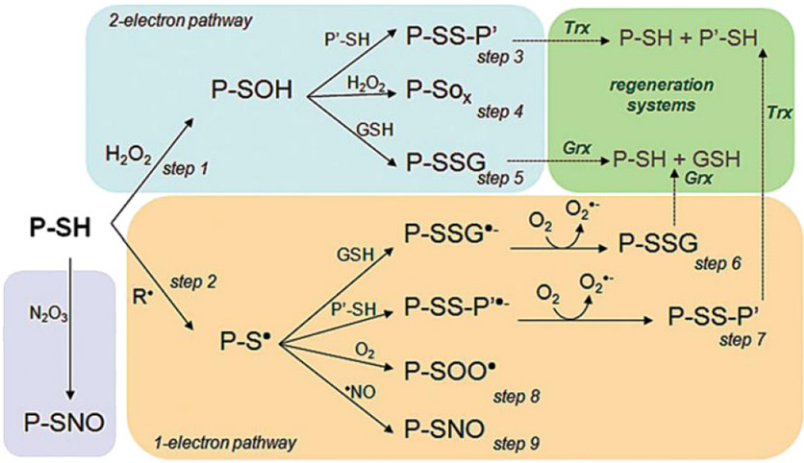

Figure 10. Following de Castro Fernandes *et al* [59]. Pathways for protein thiol oxidation. Thiols can be oxidized by 2-electron oxidants (top left panel) or 1-electron oxidants (lower right panel), both creating reactive intermediates sulfenic acid (P-SOH) or thiyl radical (P-S$^{\cdot}$), respectively. Glutathione (GSH), glutaredoxin (Grx) and thioredoxin (Trx) play various roles, as shown. Thiyl and sulfinyl (P-SOO$^{\cdot}$) radicals can propagate radical chain reactions. Nitrosylated thiols (P-SNO) can form from either thiyl radical recombination with NO (1-electron path) or by reaction with N_2O_3.

is a –SH moiety, in analogy to –OH alcohol groups in organic chemistry [70]. The only amino acid that contains –SH in its side group is cysteine, so it is usually proteins containing cysteine that are concerned with thiol-based signalling [59]. Proteins with thiol groups (denoted P-SH) often rely on them for various conformational or enzymatic functions. For example, proteins with thiol groups can form a disulfide S–S bond, thereby altering the protein folding characteristics. One factor that can greatly alter thiol group reactivity is whether the thiol group is deprotonated or ionized to form the thiolate anion P-S-. This depends on various factors, including the pKa of the group and the local environment that the protein finds itself in. For example, the H_2O_2 oxidation reactions shown in figure 7 all involve thiolates: protein tyrosine phosphatase, protein tyrosine kinase and peroxiredoxin are thiols generally in the more active thiolate state. Some idea of the complexity of RONS-thiol chemistry can be seen in figure 10, following de Castro Fernandes *et al* [59]. Note in particular the panel indicating that the protein–glutathione disulfide bond (denoted P-SS-G) that can form in either a 1-electron radical or 2-electron non-radical pathway as well as the protein–protein disulfide bonds (P-SS-P′) can both be enzymatically reduced (i.e. regenerated) by either thioredoxin (denoted Trx) or glutatharedoxin (Grx).

Figure 10 illustrates how thiols reacting with RONS achieve the necessary conditions noted above for signalling. RONS released as second messengers in the cytosol can oxidize thiol or thiolate groups on selected proteins, thereby altering their conformational and/or enzymatic properties, thus changing their cellular function in response to some extracellular stimulus. When the signal must be turned off, the reverse reactions are initiated by up-regulating Trx and/or Grx. Obviously, this is a highly over-simplified picture and many details that are in the current literature have been omitted. Nathan [71], for example, has thought deeply about precisely how RONS (he uses the terms ROI and RNI) act in signalling networks. He points out that the fact that RONS can diffuse throughout a cell and react with a class of functional groups (like the thiols/thiolates noted above) in many different molecules confers upon them a unique role. He refers to this as 'specificity of the third kind' [72]. It is worth quoting him [71]:

> . . . under physiological conditions, RNIs and ROIs react with a limited set of atoms in particular intramolecular environments. Such atoms are suitably disposed in a large number of macromolecules. This allows the RNIs and ROIs to provide a wide-reaching integrative function that ties the cell's differentiative commitments to its metabolic budget. In this way, a set of reactions can be highly prevalent and yet both chemically and functionally specific.

The discussion so far of the role of RONS in signalling has been limited to a few examples, but the number of important pathways that are known to be regulated via RONS reactions is large and growing. Nearly a decade ago (as of this writing), Nathan [72] summarized the list of signalling molecules known to be regulated by reactive oxygen and reactive nitrogen as follows: ion channels and transporters, G-protein coupled receptors, small GTPases, phosphatases, kinases, proteases, metabolic enzymes, cytoskeletal elements, translation regulators, cell-cycle control factors, transcription factors, histone (de)acetylases and DNA methylases. Via these reactions, RONS help regulate developmental processes for a suite of species, cell motility, matrix, interconnections, secretion, respiration, gene expression, metabolism, replicative cycle and apoptosis, internal biological clocks and senescence control. It seems likely that the list will continue to grow.

3.3. Electrophile products of redox reactions as signaling and anti-inflammatory agents: RONS lead to post-translational modification of proteins

As noted above, RONS generally react fairly quickly once created, forming covalent bonds with larger species. An

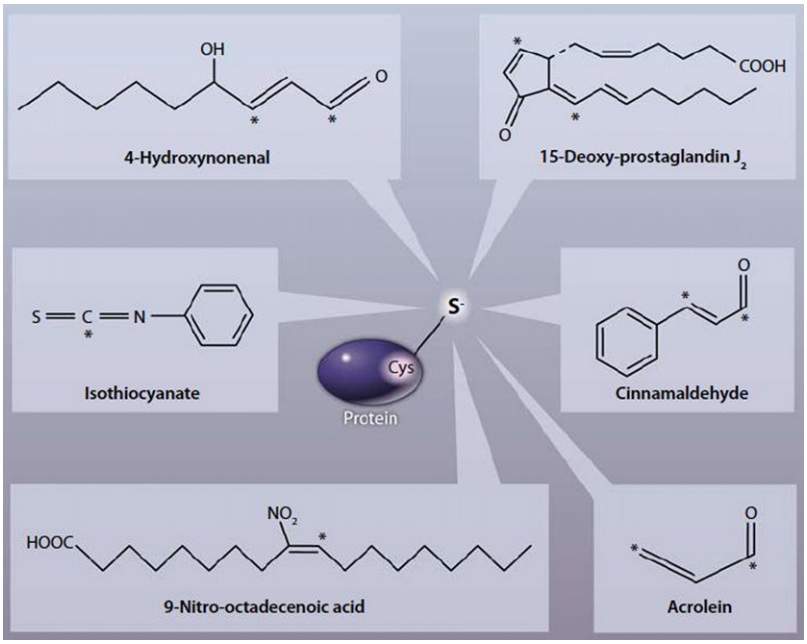

Figure 11. Following Rudolph and Freeman [73]. Chemical structures of some electrophiles. Asterisk denotes electrophilic carbon. The target thiolate containing protein is in the centre.

important class of biomolecules created by reaction with RONS is termed 'electrophilic' or simply 'electrophiles' [73]. The source of this term is that certain 'electron-loving' or electrophilic centres within these molecules prefer to react with 'nucleophilic' centres in other molecules. The key characteristic of these species is that they will react readily with the most nucleophilic amino acid of proteins, cysteine. Some examples are illustrated in figure 11, following Rudolph and Freeman [73].

The idea here is that RONS may directly react with cysteine thiols as described above, or they may react with other species to form electrophiles that in turn react with thiol-containing proteins, as shown in figure 11. Often, the electrophile product of the original redox reaction is less reactive than the RONS that created it, so the electrophilic species can diffuse farther before reacting to form an adduct with a suitable protein, lipid or carbohydrate.

Rudolph and Freeman [73] point out that reaction between electrophiles and proteins is a subset of a very important class of processes called 'post-translational modification' (PTM) of proteins. After proteins are created through the process of transcription and translation in the cell, they can be and often are modified further. A common example is phosphorylation, as discussed above in the context of H_2O_2 signalling. PTM expands considerably the number of possible proteins that can be formed in the cell (the 'proteome') over the original number encoded in the cellular DNA (the 'genome')—estimates range from one to two orders of magnitude. Reactions to form electrophile-protein adducts are examples of PTM. Of course, there are centrally important biological purposes behind such transformations in healthy cells. By allowing proteins to be altered by their environment within the cell, PTM allows protein structural changes to alter protein behaviour in response to outside stimuli. A healthy organism must react to changes induced within and without, by definition, to maintain *homeostasis*.

One of the potentially most important examples of electrophile formation from RONS is creation of nitrated fatty acids (denoted NO_2-FAs), as described by Groeger and Freeman [74]. Figure 12(*a*), following these authors, illustrates some of the reactions possible between polyunsaturated fatty acids (PUFA) and various RONS to form biologically important NO_2-FAs. Figure 12(*b*) summarizes these authors' ideas on how NO and NO_2^- (nitrite anion) interact in acidic aqueous solution. This topic is addressed in a subsequent section because of the considerable interest in the so-called nitrate–nitrite–nitric oxide pathway, and because very similar chemistry appears to result from exposure of water to atmospheric pressure air plasmas. Rubo and Radi [75] make similar points but these authors note that lipid nitration seems more associated with signalling and protein nitration with tissue damage.

The paper of Groeger and Freeman [74], as well as many other papers cited therein, explain in some detail how RONS form NO_2-FAs from PUFA precursors and how these species are intimately involved in important signalling networks. Recently, Al Ghouleh *et al* [51] have added important details to this picture of the anti-inflammatory signalling pathways associated with electrophilic fatty acids, as illustrated in figure 13. In particular, the non-enzymatic, radical chemistry path to form nitrated fatty acids (NO_2-FAs) may closely resemble the effects of plasma-generated RONS on fatty acids. The electrophilic fatty acids have three main effects as described in the figure, all resulting in the initiation of anti-inflammatory gene expression pathways.

Along these lines perhaps the most significant observation made by Freeman and co-workers for the purposes of this paper is that NO_2-FAs appear to have valuable *therapeutic* benefit. As Groeger and Freeman [74] put it,

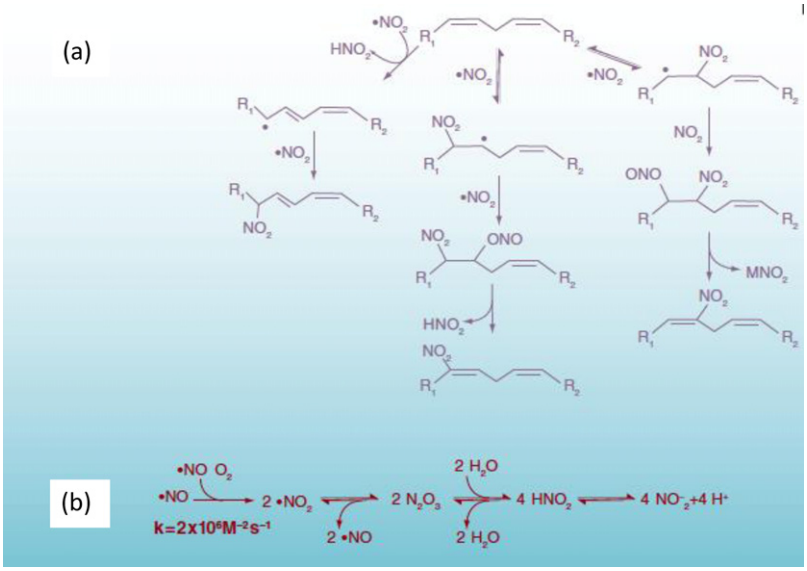

Figure 12. Following Groeger and Freeman [74]. (*a*) Mechanisms leading to the nitration of PUFA lipids via NO_x; (*b*) Reactive intermediates formed from oxidation of NO in the presence of acidified nitrite. Depending on local conditions, different reactions and products are favoured. Since all reactions are reversible, products depend on factors like the source and site of NO_x production, intermediates concentrations, and other reactions with biomolecules in the vicinity.

> For example, nitro-fatty acids induce anti-inflammatory gene expression, improve vascular function, attenuate pro-inflammatory PMN (*i.e. polymorphological nucleophils*) and macrophage functions, and, when administered in vivo, induce beneficial physiological responses such as protection from neointimal hyperplasia (*i.e. narrowing of veins*) and cardiac ischemia-reperfusion injury. This and other data support that unsaturated fatty acid-derived electrophiles hold promise as beneficial therapeutic agents—a supposition reinforced by many of the actions of dietary omega-3 fatty acids.

In short, RONS can ultimately lead to positive physiological benefit, especially in the context of immune system disorders and excessive inflammation. This insight has been expressed by others in the recent literature. For example, Hultqvist *et al* [76] in a recent review on ROS and autoimmune disorders such as autoimmune (rheumatoid) arthritis, state:

> ...there are an increasing number of findings suggesting that ROS produced by the NOX2 complex are anti-inflammatory and prevent autoimmune responses, thus challenging existing dogma. ROS might not only be produced as a mechanism to eradicate invading pathogens, but rather as a means by which to fine-tune the inflammatory response, depending on when, where and at what amounts they are produced....Hypothetically, target-specific administration of ROS-inducing substances could be therapeutic in autoimmune and other inflammatory conditions.

Of course, a natural question for plasma medicine researchers is 'can the RONS created by plasma sources applied to tissue or biological fluids/macromolecules create products that are therapeutic in autoimmune and other inflammatory conditions?' The interactions between RONS and inflammation are discussed further below in the brief summary of the immune system.

As Nathan [71] notes, there is an irony in the recent recognition that in the past RONS were thought to be exclusively hallmarks of oxidative stress but are now observed to be potent *anti-inflammatory* molecules. Nevertheless, as will be discussed in greater detail in the section on pathogenic effects of RONS, elevated RONS concentrations are observed to be associated with a vast array of disease states and ageing. Nathan [71] refers to this as '...the pathologic exaggeration of their (*i.e. RONS*) physiologic role in helping the organism coordinate its adaptive responses.'

3.4. Concentration dependence of RONS effects

The complex and often contradictory nature of the effects of various RONS—and especially NO—are difficult to fully explain. Typically, investigators note that the effects of these species will depend on the location/environment in which they are created, the time they are maintained at that location and most importantly, the concentration at which they are created. The latter effect is highlighted in figure 14, taken from Thomas *et al* [77]. As a general rule, these authors found that lower concentrations of NO tend to favour growth and act to oppose apoptosis (programmed cell death), but at higher concentrations, cell-cycle arrest, senescence or apoptosis were observed.

A similar point about the importance of RONS concentration is made by Droge [78]. His observation on the nature of RONS concentrations as a function of time is illustrated in figure 15. Droge [78] points out that temporary excursions of RONS concentration above the normal baseline level is acceptable and even physiologically desirable since RONS must on occasion increase in order to fulfil their various

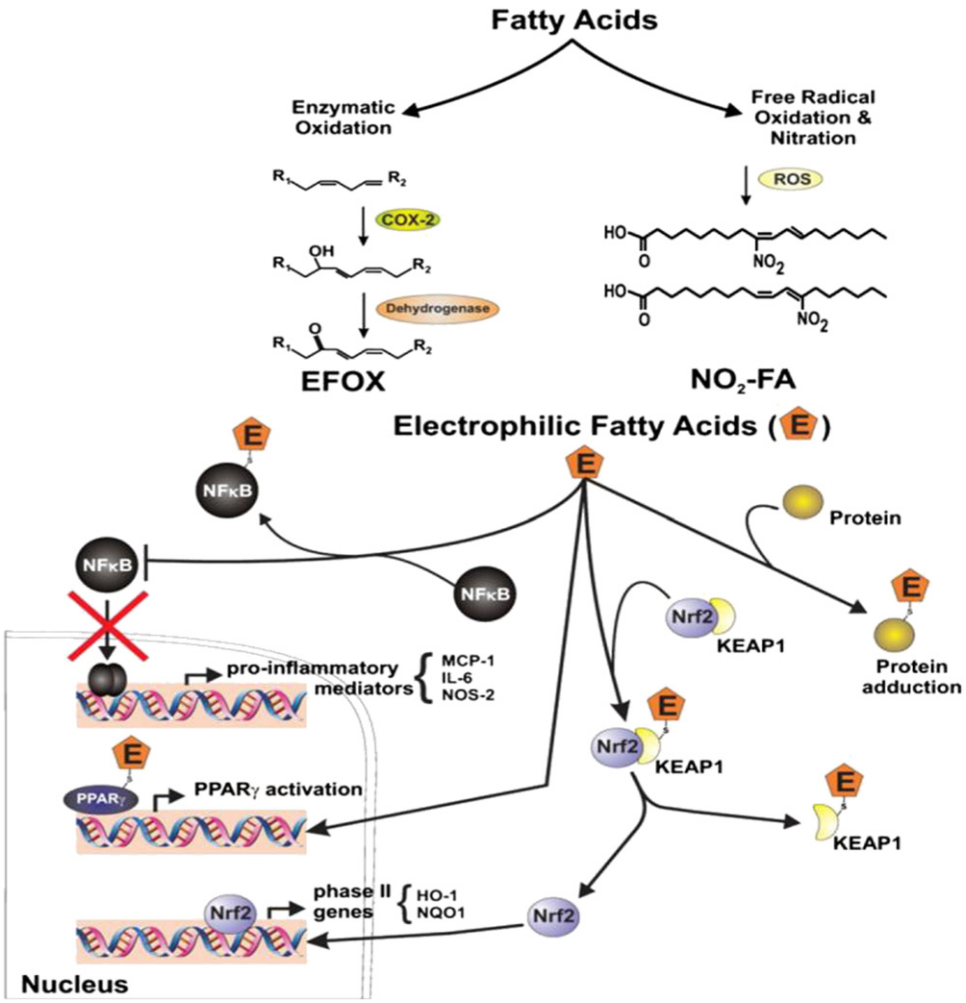

Figure 13. Following Al Ghouleh *et al* [51]. Electrophilic (e.g. nitrated) fatty acids can act as potent anti-inflammatory cell-signalling mediators. Fatty acids reacting enzymatically (left path) or non-enzymatically (right path) modulate transcription factors, regulating inflammation and metabolism. The non-enzymatic, radical chemistry path may closely resemble the effects of plasma-generated RONS on fatty acids. The electrophilic fatty acids (E): (1) adduct to specific cysteine residues on NF-kB, inhibiting pro-inflammatory cytokine expression; (2) bind to transactive downstream responsive genes; and (3) adduct to highly reactive cysteines in Keap1, releasing Nrf2,causing its nuclear translocation, binding to AREs, and activating phase II genes. All of these actions correspond to anti-inflammatory pathways.

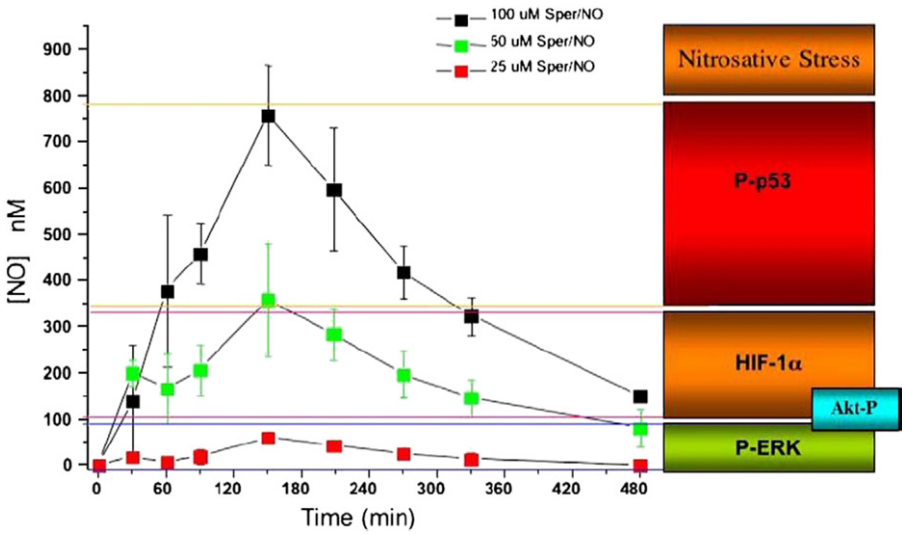

Figure 14. Following Thomas *et al* [77]. Plot shows how differing concentrations of nitric oxide over about the same time period of about 8 h drastically affected the biological result, from cell signalling at the lowest concentration to nitrosative stress at the highest.

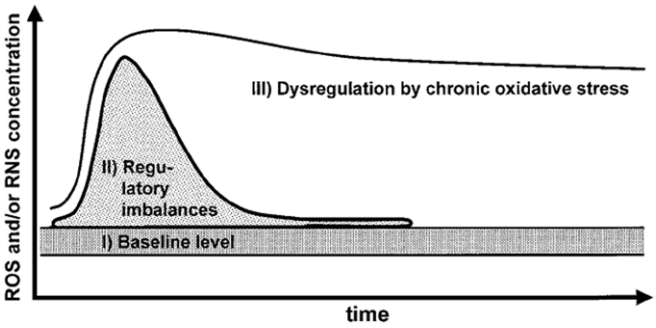

Figure 15. Following Droge [78]. The baseline level of RONS can be exceeded temporarily in a way that acts to regulate some cell function. Normally, the RONS concentration returns to baseline after this excursion and no harm is done. If, however, the RONS concentration does not return to an appropriate baseline, pathological conditions result.

physiological missions. However, if it turns out for some reason that these species do not return to baseline and remain at an elevated level for an extended period, then pathological effects are to be expected. This can happen, for example, if inflammation becomes chronic, as discussed in the following section.

3.5. Immune system

The purpose of the immune system is to recognize pathogenic organisms (distinguishing 'self' from 'non-self') and clear infections and tumours [28, 79]. Vertebrate immune systems combine two complementary immune systems: a rapid response, but non-selective 'innate' part and a slower and much more selective 'adaptive' or 'acquired' part. Bogdan *et al* summarize the major RONS enzymatic generation pathways and associated microbial resistance genes [28]. The adaptive immune system is the one that gets most of the attention, with its antigens, antibodies, B cells and T cells, not to mention its role in enabling all-important vaccines. Its importance is uncontested, but the innate system is important too. These systems are known to be coupled and one of the ways they interact is through RONS. For example, Droge [78] highlights the way that ROS generated by the innate immune system sensitize and amplify T lymphocytes, active in the adaptive immune system.

The innate immune system is associated with inflammation and RONS play key roles in this system, both as anti-pathogenic compounds and in the complex signalling networks that control the process. As noted by Nathan and Ding [21], inflammation is a frequent occurrence in humans because '. . . we are partly microbial and we move in a microbial world.' Nathan and Ding [21] stress that RONS have a widespread, pervasive effect on both promoting and resolving inflammation.

Indeed, in some ways the most important issue with inflammation is the need to terminate it fairly quickly since the non-specific anti-infective actions—mostly release of RONS—can be very damaging to the host. By some estimates, the damage done by chronic, unresolved inflammation is greater than pathogen-associated damage. Non-resolving inflammation is associated with many of the most serious and widespread of modern diseases, including atherosclerosis, diabetes, asthma, arthritis, cancer and even obesity. The potential importance of the observations noted above on the ways that RONS or RONS reaction products might act to *resolve* inflammation seems clear.

Figure 16 illustrates the basic events and timeline in the innate immune inflammatory response [79]. The innate immune system responds to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide from the outer membrane of gram negative bacteria like *E. coli*. Receptors activated by the presence of PAMPs initiate the inflammatory response by first recruiting neutrophils, which are specialized forms of leukocytes. These cells engage in the so-called 'respiratory burst,' in which O_2 in solution reacts enzymatically via phagocyte oxidase (also called NADPH oxidase) with NADPH to form $NADP^+$ and O_2^-, or superoxide anion. O_2^- then goes on to play important roles in a series of reactions, as illustrated, for example, in figure 8.

Another important class of leukocyte, the macrophages, is attracted by the release of pro-inflammatory cytokines released by neutrophils. Macrophages engulf the pathogens and kill them by exposing them to RONS (mostly O_2^- and NO). NO is created by both macrophages and neutrophils through the enzymatic reaction of inducible nitric oxide synthase (iNOS; also called NOS2) acting on L-arginine. Some of the reactions between RONS that are relevant to the immune system are illustrated in the sequence presented in figure 1. Generally, the enzymatically created originating species O_2^- and NO react with each other and other species to form a whole suite of anti-pathogenic products, including $ONOO^-$, NO_2, NO_2^-, NO_3^- and HOCl. The last compound (hypochlorous acid; known to react widely with biomolecules) is thought to result from reaction between H_2O_2 and Cl^- and involves as well the enzyme myeloperoxidase [28, 78]. The immune systems of different species may use RONS differently against the same organism. For example, it is known that mouse macrophages attack mycobacterium tuberculosis with RNS but human macrophages may not [80].

A relatively recent, and still somewhat controversial observation, is that antibodies can catalyze the generation of H_2O_2 and O_3 and that these species can play key microbicidal roles when antibodies coat neutrophils [81–83]. The difficulty in unambiguously identifying the reaction products of O_3 have led to some doubts about this mechanism [84]. More recently, Yamashita *et al* [85] reported that amino acids could themselves catalyze $^1O_2$ and water 'to an oxidant with the chemical signature of ozone,' and that this amino-acid catalyzed oxidant 'showed bactericidal activity in human neutrophils.'

Macrophages ideally begin to slow down or 'resolve' the inflammatory state by secreting anti-inflammatory cytokines, as shown in figure 16(*a*). But as discussed previously, it is also possible for a state of non-resolved, chronic inflammation to appear, and it is known that this unhealthy situation can be caused or exacerbated by pathogens themselves [79].

In summary, RONS have been shown to play several roles in fighting infection in both the innate and adaptive immune systems, mostly through signalling, amplification

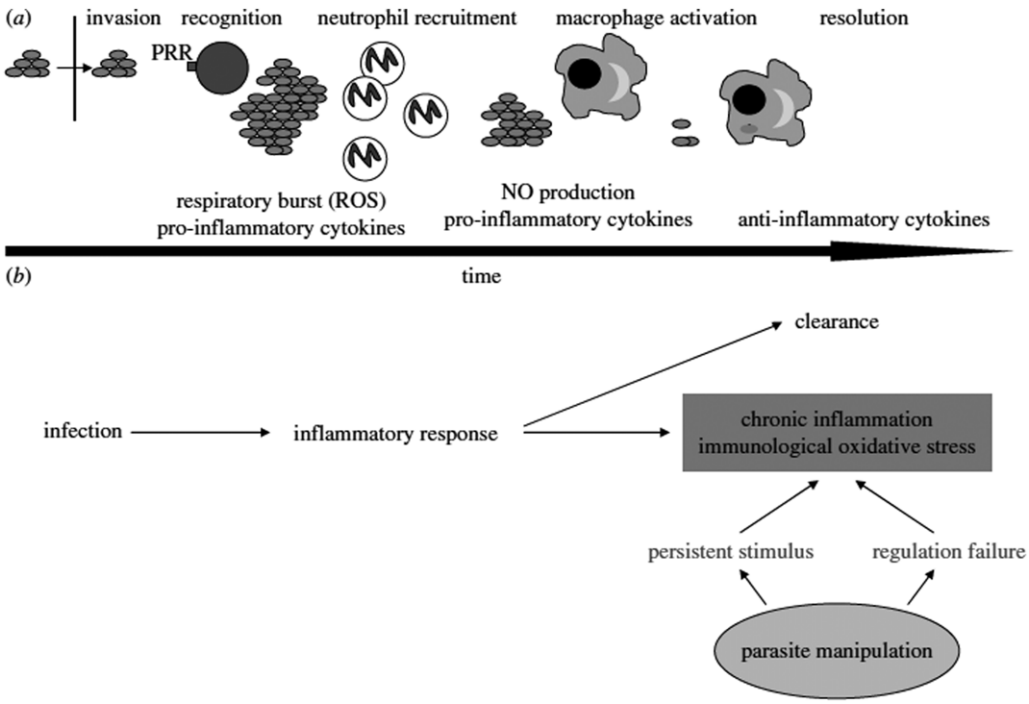

Figure 16. Following Sorci and Faivre [79]. (*a*) Time course of acute inflammation: pattern recognition receptors (PRRs) on cell membranes bind to pathogen molecular signatures upon pathogen entry into host, followed by neutrophil recruitment and subsequent respiratory burst. Macrophages are activated by sustained production of pro-inflammatory cytokines and initiate the inflammation resolution phase after encapsulating pathogens by secreting anti-inflammatory cytokines. (*b*) Two outcomes of inflammation: (i) clearance and resolution and (ii) chronic inflammation with its associated oxidative stress. Parasites are able to interfere with inflammatory response to improve survival.

and anti-pathogen cytotoxicity. There is evidence that RONS act synergistically with each other and/or with other antimicrobial systems so pathogens have difficulty in avoiding their combined effects [86]. However, it is also known that pathogens have evolved a variety of strategies to minimize the effects of RONS, including developing resistance genes [28]. Several authors point to the long history of host-pathogen 'arms races' in which each side seeks to gain an advantage [79, 86, 87]. Nathan [21] points out that RONS have a 'pervasive influence' on both promotion and resolution of inflammation. Indeed, the possible role of RONS to help resolve inflammation is intriguing from the point of view of plasma medicine and should be identified as a key direction for the field.

3.6. Wound healing

Wound healing follows tissue injury in a series of overlapping and orchestrated processes, starting with haemostasis (stopping blood loss and formation of a fibrin clot), inflammation, proliferation and finally remodelling [88]. The inflammation phase involves the actors discussed above—namely, neutrophils and macrophages. In the case of wound healing these inflammatory cells play a key role in releasing so-called growth factors that act to attract fibroblasts, keratinocytes and epithelial cells, promote the formation of the extracellular matrix and prompt angiogenesis. Blood vessel growth is obviously essential in order to supply the healing region with oxygen and other nutrients. Wounds that fail to heal normally are termed 'chronic' and are a major problem in the developed world as they are associated with obesity and diabetes [8].

RONS are now known to be key players in wound healing [89–92]. First, they are known to be essential for the initial stage of haemostasis, through their roles in mediating tissue factor (TF-mRNA), platelet recruitment and platelet activation [92, 93]. As noted above, inflammation is intimately involved with RONS in their roles with neutrophils and macrophages. H_2O_2 is also known to be active as a second messenger for platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and tissue growth factor (TGF) [92, 94].

Nitric oxide is arguably the most important RONS involved in wound healing [90, 92, 95, 96]. Figure 17 [95] shows an approximate time history of NO released during wound healing. It obviously follows the inflammation phase fairly closely. Luo and Chen [96] attribute the role of NO in wound healing to its functional activities in 'angiogenesis, inflammation, cell proliferation, matrix deposition and remodelling.' These authors suggest that future developments in human gene therapy may be exploited to deliver the NO-creating enzyme nitric oxide synthase (NOS) in such a way to counter chronic wounds. The topic of using exogenously delivered NO, including from plasma sources, is addressed in a subsequent section.

3.7. Non-enzymatic nitrate, nitrite and nitric oxide

Nitric oxide is created enzymatically, as noted above, through three isoforms of NOS: endothelial NOS operates in endothelial tissue; neuronal NOS in neurons and inducible

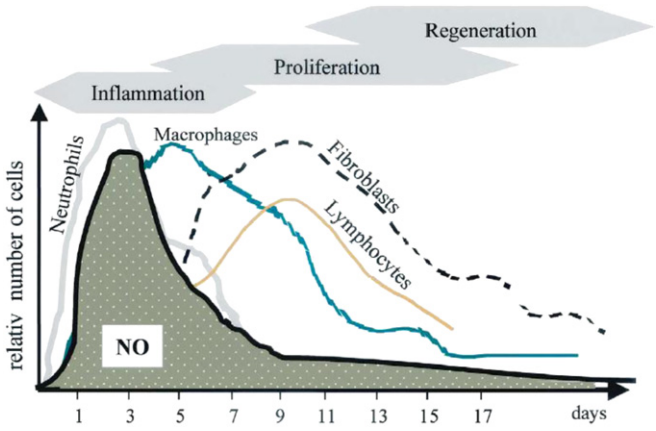

Figure 17. Following Witte and Barbul [95]. Phases of wound healing and generation of wound NO, primarily during the inflammation stage, and most closely associated with neutrophils.

NOS in immune cells. But there is another important pathway to create NO and related compounds that comes from the diet. As illustrated in figure 18 [97], mostly nitrate (NO_3^-) and some nitrite (NO_2^-) in the form of inorganic anions are ingested from eating certain foods, such as leafy green vegetables. Commensal bacteria in the mouth reduce NO_3^- to NO_2^-, and the resulting nitrite, when swallowed, is mixed with highly acidic gastric juices, releasing NO and other nitrogen oxides. (see figure 12(*b*)) The remaining NO_3^- and NO_2^- is either absorbed in the intestine, destined to enter the bloodstream, or is eliminated via the kidneys. The nitrate in the blood is concentrated in the saliva and the process continues. This process results in mM nitrite concentrations in the saliva. As Lundberg *et al* [97] point out, this reductive cycle (NO_3^- to NO_2^- to NO) has been observed in the oral cavity, on skin and in urine. In general, acidified nitrite is associated with the formation of NO and other related compounds. In all cases, the resulting NO_x is thought to provide significant antimicrobial protection. For example, it is suspected that antimicrobial salivary NO_x is the reason animals lick their wounds. The presence of NO_x has been shown to increase both mucosal blood flow and mucus generation in the gastrointestinal tract [98].

It has been known for some time that breast milk contains nitrate and nitrite, but recent research has shown that human breast milk contains significantly more nitrite than nitrate in the first 3 days after birth ('colostrum') than in the milk provided by the mother after that, as illustrated in figure 19 [99]. It is speculated that this serves to provide more NO in the baby's stomach in the first few days after birth since the baby has not had time to acquire the mouth and stomach bacteria that convert nitrate into nitrite, and hence to NO/NO_x in the stomach, mouth, skin and urine. The net effect for the vulnerable newborn seems to be to enhance protection against infection. This study highlights the likely important role of the nitrate/nitrite/NO system in promoting neonatal health.

One of the most important aspects of this pathway is thought to be the fact that nitrite (NO_2^-) will release NO and other RNS in acidic, hypoxic environments. It therefore serves as a kind of NO reservoir in the body. Thus, NO_2^- is thought to protect against a number of problems, ranging from ischemia-reperfusion injury (in heart attack and stroke, for example) to kidney injuries and hypertension [100].

Concerns about the formation of potentially carcinogenic N-nitrosamines from dietary NO_2^- and NO_3^- are now thought to be exaggerated, especially considering the relatively high concentration of these anions in the body from diets associated with health, not disease [101]. The use of nitrite as a drug is addressed in the section addressing RONS therapeutics.

3.8. The dual nature of RONS in homeostasis and disease: the example of RONS, insulin and diabetes

To conclude this section on normal homeostatic, physiological processes associated with RONS, it may be appropriate to look at a specific example. In order to illustrate the invariably dual physiological/pathological nature of RONS, the situation with respect to insulin, diabetes and RONS is helpful. Bashan *et al* [102] in a recent review observe that both positive and negative regulation of insulin signalling occurs due to RONS. RONS therefore act as a 'double-edged sword' in modulating insulin signalling, as summarized in figure 20. The presence of insulin will increase RONS rates of creation, and it is known that RONS are important cellular second messengers that allow insulin to perform its proper physiological functions. But it is also true that RONS appear to be associated with both cellular and whole body resistance to insulin. RONS apparently alter proteins that are known to be important as insulin signalling molecules or otherwise affect in a deleterious way insulin signalling pathways. In addition, transcription factors are altered as well as hormones and cytokines that indirectly affect insulin sensitivity [102].

How to sort out the physiological from the pathological roles of RONS and then exploit this understanding to develop effective therapies? Bashan *et al* [102] write that this is especially difficult for RONS for three reasons: (1) There are many different RONS, and although they interact and affect each other in complex ways, they are certainly not acting in the same way; (2) RONS are relatively short-lived species and are difficult to measure reliably and (3) Nature has worked out a multifaceted system to control RONS and its inner workings are far from apparent. There is, therefore, a kind of 'biological context' within which RONS function either for good or ill and all that can be said at present is the rather reductive fact that when things are in a proper balance, RONS are functioning to maintain homeostasis and when not, they seem to be part of the disease manifestation. Attempts to re-establish an oxidant–antioxidant balance for therapy have been disappointing to date. At the current time, this summary of RONS, insulin and diabetes, with a few exceptions, seems to be representative of the situation with RONS for many of the diseases with which it has been implicated. More about the correlation of RONS and disease is presented in the following part of the paper. Nevertheless, in spite of this relatively gloomy perspective, there are clear opportunities for therapy, as noted previously. For example, the clearly anti-inflammatory roles played by nitrated fatty acids and the other anti-inflammatory effects of RONS or electrophiles created from RONS on ameliorating afflictions associated with autoimmune disorders

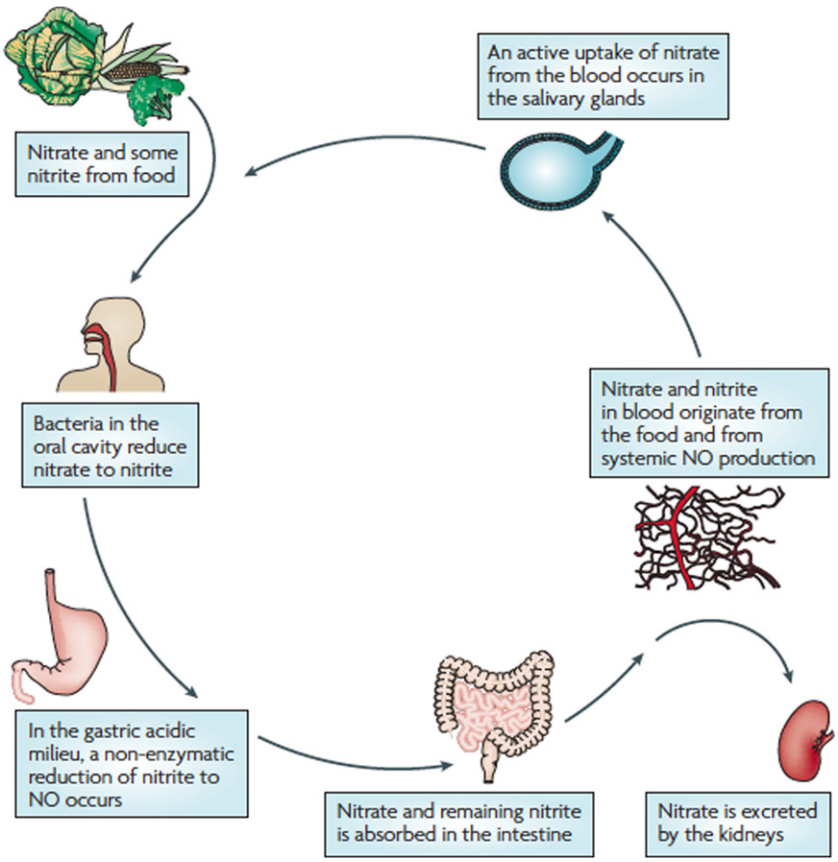

Figure 18. Following Lundberg *et al* [97]. The entero-salivary circulation of nitrate in humans. Inorganic nitrates from diet are rapidly absorbed in small intestine, about 25% of which ends up in saliva. Commensal oral bacteria reduces nitrate to nitrite, which when swallowed is converted to NO and other nitrogen oxides in the acidic gastric juice. Nitrate and nitrite absorbed into blood and can serve as a source of NO in blood and tissue under low O_2 (hypoxic) conditions.

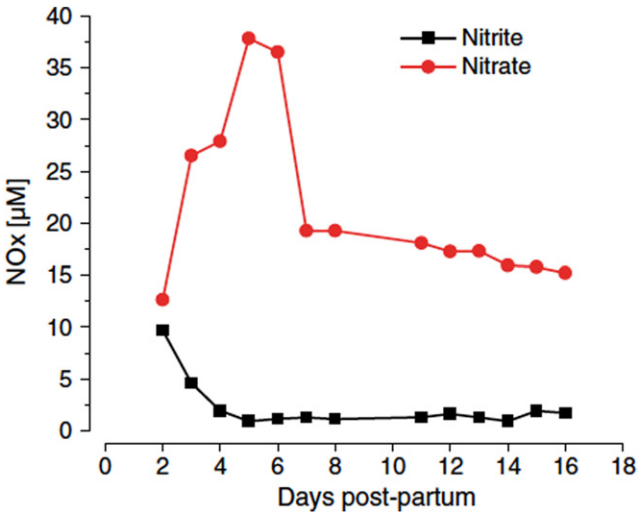

Figure 19. Following Berens and Bryan [99]. Breast milk from nursing mothers over 16 days were averaged, showing how NO_2^-/NO_3^- concentrations peak shortly after birth, suggesting neonate's need for extra NO_2^- in milk to provide additional antimicrobial protection.

offer promise. Other promising avenues are associated with cancer therapy, wound healing, infectious disease therapies and other applications, as discussed in the section following the next one.

4. Pathophysiological effects of RONS

4.1. Introduction

The pathophysiological and toxic effects of reactive oxygen and nitrogen species and the associated concepts of oxidative and nitrosative stress are widely documented in the current literature and some of the history of these ideas mirrors what was described in the previous section. The comprehensive monograph by Halliwell and Gutteridge—arguably the 'bible' in the field—is especially useful on this and related topics [6]. Overviews and reviews of oxidative stress, including how the concept has evolved are provided by Roberts *et al* [103], de Castro Fernandes *et al*, [59], Kulkarni *et al* [104] and Jones [105, 106].

In the interest of brevity, this section focusing on RONS-associated pathophysiology will only summarize a small part of this voluminous literature (with a very limited set of associated references) without trying to describe proposed mechanisms in any detail. In fact, in many cases, the literature primarily establishes a correlation between RONS, RONS reaction products and associated disease states or ageing. Strict cause-and-effect relations, at least in a way that offers a clear path to therapy, are rarely identified. The most common observation is that markers of oxidative and nitrosative stress are obviously and often copiously present, but simple strategies

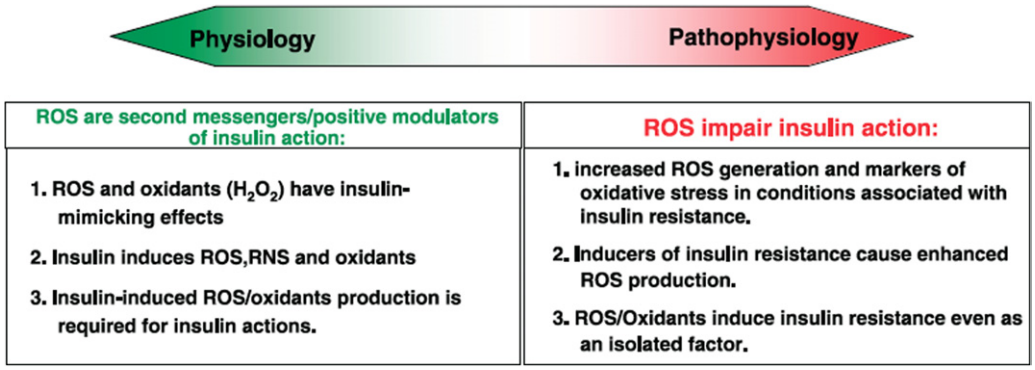

Figure 20. Following Bashan *et al* [102]. RONS can exert both physiologically positive effects on insulin action, but also negative, pathological effects as well. The complexity of RONS has made development of effective RONS-based therapies difficult to develop.

like adding known antioxidants rarely are effective and are sometimes distinctly counter-productive [61]. The general conclusion is usually to note that RONS can play either positive or negative roles but that the complexities of the networks generally preclude the obvious and straightforward interventions.

The reviews of Valko *et al* [107] and more recently Chiurchiu and Maccarrone [108] provide excellent summary overviews of the dual character of RONS in a variety of disease states and in normal physiology. Earlier views on the role of RONS in disease was summarized by Cross *et al* [109]. These authors address the roles of RONS in cancer, cardiovascular disease, hypertension, ischemia/reperfusion injury, diabetes, skin diseases, neurodegenerative diseases (NDs), autoimmune diseases and ageing, among others. Each of these topics could easily be treated in a multi-volume series of monographs; furthermore, the list of topics chosen and discussed below is only the briefest of summaries.

4.2. Cancer

As the title of a recent best-selling book announces, cancer can be thought of as the 'emperor of all maladies,' so perhaps it makes sense to start with it [110]. There is considerable evidence from many sources linking chemical carcinogenesis to the creation, and especially the prolonged creation, of oxidants in the cell, leading to DNA damage and mutation as well as alterations to gene expression [111]. It has been widely observed that, although apparently contradictory, *in vitro* antioxidant addition often suppresses tumour formation and growth, but enhancing antioxidant mechanisms has not been a successful anti-cancer therapy *in vivo*. Antioxidants commonly lead to chemoresistance and a generally poor prognosis.

4.3. Cardiovascular disease (CVD)

CVD is obviously another extremely important disease in the modern world and there is a lot of evidence linking the entire set of CVD with imbalances in RONS. This set of diseases includes coronary artery disease, hypertension, congestive heart failure and stroke. They represent the leading cause of death and disability in the developed world (e.g. Pacher *et al* [66]).

4.4. Neurodegenerative disease

All NDs involve damage or degeneration of neurons, or nerve cells, that transmit information via electrochemical sensing. The most important NDs are Alzheimer's, Parkinson's and Huntington's diseases and amyotrophic lateral sclerosis (ALS). Also included in this category sometimes are multiple sclerosis (MS), prion disease and Friedreich's ataxia, although MS is generally thought of as an inflammatory disease, prion disease is essentially infective, and Friedreich's ataxia is a relatively rare genetic disorder. RONS are implicated in all of these NDs.

4.5. Diabetes mellitus, metabolic syndrome and the effects of obesity

Diabetes mellitus (generally just 'diabetes') is a metabolic disorder that is characterized by high levels of glucose (hyperglycemia) and is due to inadequate creation or action of the hormone insulin. Metabolic syndrome is a combination of medical problems that increase the risk of contracting diabetes and CVD. The effects of obesity are included here in part because of the obvious metabolic connection to diabetes and metabolic syndrome, plus the fact that obesity is a key predictor of both diabetes and CVD. All three are associated with elevated and/or poorly regulated levels of RONS.

4.6. Lung

The lungs are obviously subjected to a steady exposure of oxidative stress, not only from O_2 but also from pollutants such as O_3, NO_x, tobacco smoke and other combustion products, radical-generating particles (e.g. silica) and other sources. Many lung diseases are thought to be caused by or are exacerbated by exogenous RONS, RONS-generating substances, as well as endogenous sources associated with the immune system. Disorders that are thought to be caused by RONS include silicosis, acute respiratory distress syndrome, bronchial asthma, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and some sleep disorders. They may also be important in cystic fibrosis, lung cancer and pulmonary sarcoidosis.

4.7. Skin

Similar to lungs, skin is also exposed to many RONS-related attacks, both direct and indirect. Solar ultraviolet radiation is known to generate RONS in skin through induction of nitric oxide synthase 2, one of the enzymes responsible for NO creation. RONS are known or suspected to be associated with chronic skin inflammation and other skin disorders such as psoriasis, atopic dermatitis, acne, vitiligo and contact dermatitis. Skin cancer may be related to RONS as well.

4.8. Reproductive disorders

RONS are known to be important factors in both male and female fertility and infertility. In male infertility, RONS are implicated in lipid peroxidation and DNA damage, among other things. Female reproduction is strongly affected by RONS, and NO in particular. RONS and oxidative stress are thought to be involved in abortions, preeclampsia, hydatidiform mole, fetal embryopathies, preterm labour and gestational diabetes [112, 113].

4.9. Inflammatory bowel disease

IBD include ulcerative colitis and Crohn's disease, and other inflammatory conditions of the colon and small intestine. Considerable evidence points to a role played by RONS in this set of diseases, probably related to problems with the mucosal immune system and its interactions with resident bacteria [114, 115].

4.10. Autoimmune disorders

Rheumatoid arthritis, systemic lupus erythematosus, psoriasis and celiac disease are all autoimmune disorders linked with RONS. Autoimmune disorders result from an overactive immune system acting against tissue and substances normally in the body. Excessive oxidative stress is associated with these diseases, but they are complex disorders with both genetic and environmental components. One aspect that is characteristic of these diseases is inefficient clearing of dying cells, possibly leading to autoantibodies and autoimmune reaction [108].

4.11. Role of iron

Iron plays a special role in the interactions of RONS due to its potentially catalytic nature. Iron is generally in the form of a relatively unreactive ligand within an organometallic complex, but may be released as free iron. This 'unregulated' iron can participate in catalytic cycles in which normally fairly innocuous hydrogen peroxide (H_2O_2) can react to form strongly damaging OH radical via reaction with Fe^{2+}, also known as 'Fenton's reaction.' The Haber–Weiss reaction reduces the oxidized Fe^{3+} state to the Fenton-active Fe^{2+} state by reacting with O_2^-, perpetuating the catalytic cycle. Kell [116, 117] has extensively documented the association of free iron with a vast suite of diseases (see figure 8(*b*)).

4.12. Ageing

The free radical theory of ageing, first proposed by Harman in 1956 as noted in the introduction, posits that the ageing process results from the accumulated damage caused by RONS, and especially ROS [11]. Studies over the years have tended to show, however, that although the presence of oxidative damage certainly increases generally with age, there is no direct cause-and-effect relationship between 'excess' RONS and the ageing process. Nevertheless, the association of RONS with many degenerative diseases continues to suggest connections between RONS and ageing. As noted above, Lapointe and Hekimi [13] point out that the mitochondrial free radical theory of ageing has been arguably refuted in its strict form that views ROS as *the* cause of ageing, although there does appear to be a strong association or correlation of ROS and oxidative stress with age-related diseases and disorders. Counter-intuitively, the latest research suggests that low levels of oxidative stress may allow organisms to adapt in a way that *extends* longevity. For example, in a recent review, Ristow and Zarse [16] assert that caloric restriction, glucose restriction and exercise are linked through a common metabolic feature, namely, citing an '... increased mitochondrial metabolism and ROS formation inducing an adaptive response that culminates in increased stress resistance, antioxidant defense and extended life span.' These authors describe this effect as an example of 'mitohormesis,' or hormesis associated with the mitochondria, and their idea is illustrated in figure 21. The term 'hormesis' refers to the principle that a compound may be toxic in large doses but therapeutic or beneficial (perhaps through an adaptive response) in smaller doses. The idea has been promoted by Calabrese for many years [118]. The concept has become a popular theme recently with respect to the health- and longevity-assisting properties of oxidative stress and ROS. It appears that the increase in ROS levels associated with exercise, glucose or caloric restriction induces an adaptive response and leads to increased lifespan, at least for certain experimental animals. Antioxidants that inhibit ROS increase will eliminate this mitohormesis and no life extension is observed.

Hekimi *et al* [17] (see figure 22) illustrate the principle for worms (C. elegans) and rodents: longer lifespan is associated with elevated ROS and/or oxidative stress. These authors offer an intriguing and seemingly compelling theory to explain the observations that ROS are associated with age-related diseases but ROS are also correlated with life extension. Figure 23 summarizes their hypothesis: organisms use ROS as signalling agents to elicit a stress response when ROS-independent damage is detected. When the organism is young, little age-dependent (non-ROS induced) damage has accumulated and the signalling ROS pose no problem as the organism has effective ROS detoxification mechanisms. However, as the organism ages and age-related damage had begun to accumulate, when similar ROS-independent damage is detected and the organism releases signalling ROS to initiate the stress response to deal with the damage, the signalling ROS themselves begin to add to the damage. This can lead to a destructive positive feedback: more damage leads to more ROS release, but insufficient ROS detoxification means

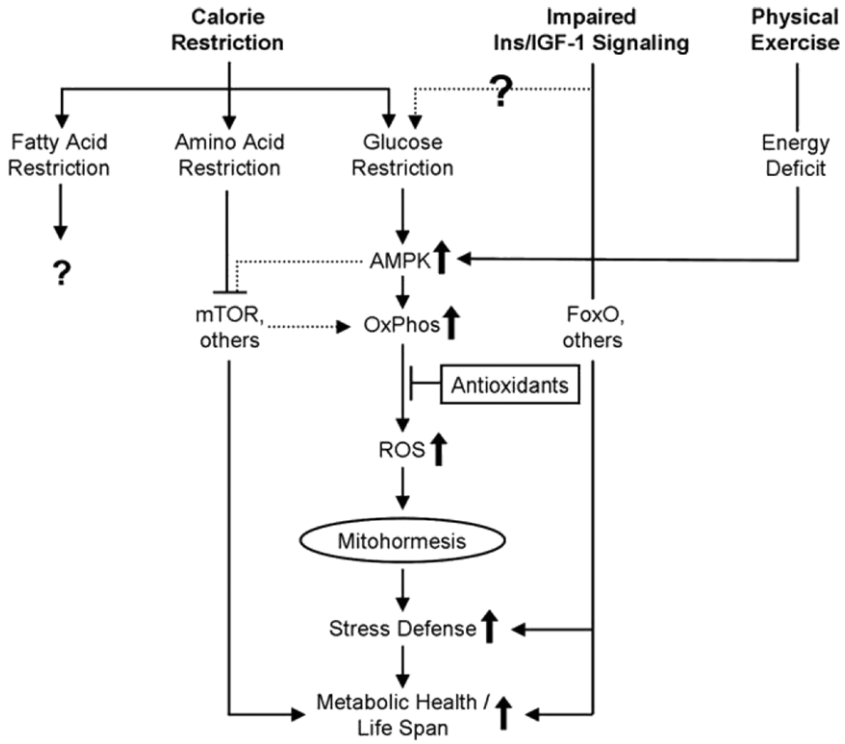

Figure 21. Following Ristow and Zarse [15]. Mitohormesis and life extension: calorie restriction and exercise both require induction of mitochondrial metabolism to result in life extension or other health benefits. Mitochondrial metabolic increase is associated with an increase in ROS and this in turn is required to induce adaptive response and increased lifespan. Antioxidants that inhibit ROS increase block mitohormesis and life extension. Impaired insulin-IGF-1 signalling connection to ROS not yet demonstrated.

these ROS begin to add to the damage. This is consistent with the observation that age-related damage seems to lead to ever-increasing levels of damage—damage catalyzes further damage [119].

5. Reactive species therapeutics

5.1. Introduction

As we have seen in the previous sections, RONS and other reactive species (RS) are both integral to normal physiology and clearly involved (directly or indirectly) in most major disease states known to humans. But can they act as therapies in themselves or as part of other therapies? The answer appears to be 'certainly yes,' and this section aims to outline and summarize some of the evidence, especially with an eye towards the implications for plasma-based medicine and therapy. The first two sections will focus on *cancer therapy* and *infectious disease therapy*. The subsequent section switches orientation and rather than focusing on a single disease, discusses the significance of inorganic nitrites and the closely related gaseous nitric oxide, in various therapeutic modalities. Following this, a short summary of the use of ultraviolet radiation in therapy, especially in dermatology, is presented with an emphasis on the role of photo-generated RONS. The general order of discussion will be to highlight and summarize existing therapies that rely in whole or in part on various RS (mostly RONS), then present emerging reactive species therapies, then finally to note the relationship with emerging or potential plasma-based RS therapies.

The final part of this section addresses some emerging or controversial technologies that employ RS, including ozone (O_3) and low-level light therapy (LLLT).

5.2. Cancer therapy

5.2.1. Radiation therapy (radiotherapy). Radiation therapy (sometimes referred to as 'radiotherapy') has traditionally involved the use of ionizing photons (e.g. x-rays) directed at tumours for their elimination. A more recent development, not discussed further here, is the use of energetic particle beams. Barnett *et al* [120] note that up to 50% of all cancer patients worldwide receive some form of this therapy. It has been known for a long time that ionizing radiation in aqueous solution creates radicals such as OH, H, HO_2 and so forth (e.g. Gerschman *et al*, [56]). The anti-tumour effects of radiation consist of direct attack on the DNA of cells from the energy deposited there by photons, and the indirect attack from the radicals generated. It is now understood that many fewer radicals are created by ionizing radiation than are normally generated within the cell by various metabolic processes [121]. Mikkelsen and Wardman [122] note that cells appear to *amplify RONS* in response to ionizing radiation, and that these secondary RONS species are the ones primarily responsible for the biochemical effects of radiation therapy. This seems related to the so-called 'bystander effect,' well known in radiation therapy. The evidence has long pointed to radiation effects on cells that are clearly peripheral to the main radiative energy deposition and associated radical creation, suggesting some kind of transport to adjacent cells of information. Recent

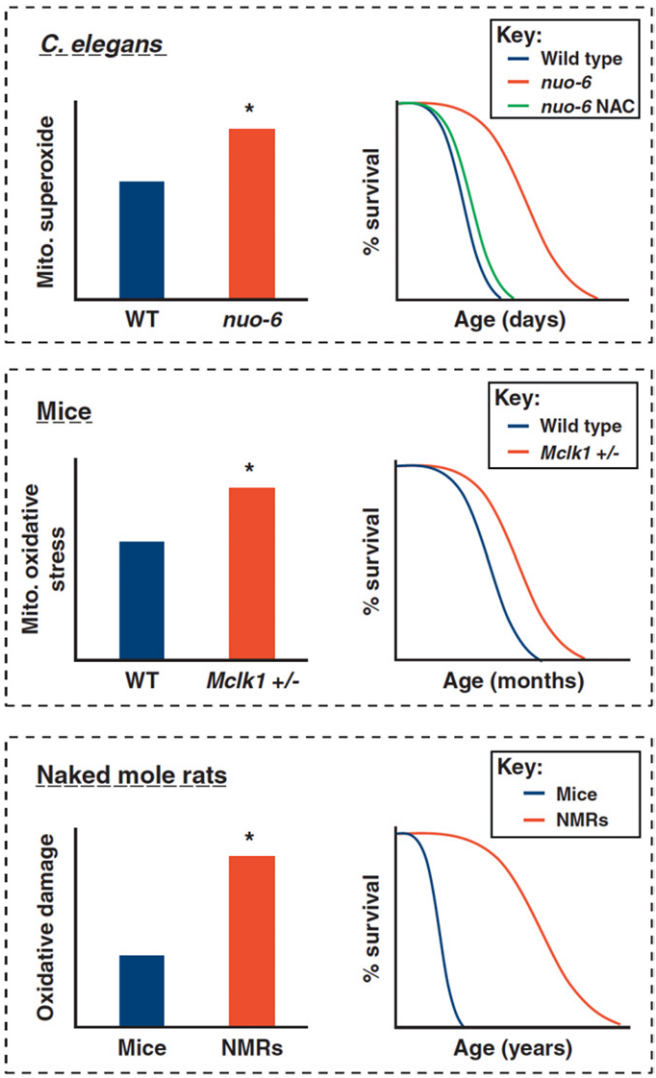

Figure 22. Following Hekimi *et al* [17]. Summary of studies showing high ROS corresponds to lifespan extension. Top panel: mutants (nuo-6) of C. elegans worm produce more O_2^- and live longer than wild type unless antioxidant (NAC) is added. Middle panel: Mutant mice (Mckl1) show higher mitochondrial oxidative stress but live longer than wild type mice. Bottom panel: naked mole rats (NMR) show much higher oxidative damage than normal mice but live longer. (Plots are qualitative only; asterisk indicates statistical significance.)

studies of RONS signalling in plants (discussed below) show that signalling can occur in propagating waves over significant distances [123].

Mikkelsen and Wardman [122] discuss another important point that is relevant to the use of plasma-generated reactive species. These authors conclude that cells convert oxidative stress signals into nitrosative signals because of high ROS reactivity. Their idea is that ROS are initially produced by radiation, but RNS are the subsequent effectors and activators of this signalling information. In part, this is due to the fact that lipophilic species NO and NO_2 may concentrate in the hydrophobic domains of membranes, creating the reactive species N_2O_3. A likely target, according to these authors, are proteins with tyrosine moieties, resulting in protein tyrosine nitration. The question of how species created in a plasma, often including both ROS and RNS, translates into a biological effect is far from understood but is clearly important for the technology. It may be that a similar coupling between ROS and RNS occurs in the case of plasma–cell interaction as well.

5.2.2. Photodynamic therapy. As Dolmans *et al* [124] point out, light has been known to have therapeutic potential for thousands of years, going back to the time of the ancient Egyptian, Indian and Chinese civilizations. The modern medical uses began in earnest in the 1960s. PDT is being tested clinically for use in various cancer treatments, a wide variety of dermatological applications, and is an approved technology for age-related macular degeneration and other eye diseases [124]. It is also currently approved for use in the US and several other countries for early stage malignancies and for the palliation of late-stage tumour symptoms [125].

As illustrated in figure 24, PDT works using a photosensitizer (common early photosensitizers were porphyrins) that must selectively adsorb in the tissue to be treated [124]. Finding good photosensitizers is key to the technology—they must be selective to desired tissues, have little or no intrinsic toxicity and be sensitive to light. A visible light source is then used to create an excited form of the photosensitizer. This excited state can react directly with cell molecules such as the cell membrane, creating a radical site by removing a hydrogen atom in what is termed a type I reaction. This radical site can then react with O_2 in solution to create the excited states species $^1O_2$. This excited O_2 is then the active agent in oxidizing various substrates. Alternatively, the excited photosensitizer can create $^1O_2$ directly from O_2 in solution; this is termed a type II reaction. A similar definition of type I versus type II excitation is discussed below in the context of ultraviolet radiation effects on tissue.

Since the light does not penetrate far into tissue, PDT creates these species near the surface—typically within several millimetres. The fact that PDT creates ROS locally near the surface and that these species appear to act in a therapeutic fashion makes this technology appear similar to plasma-generated RONS that are generally created at the surface of a biological target.

Mroz *et al* [126] note that PDT will directly kill tumour cells by necrosis and apoptosis in a local manner, and PDT is also known to shut down tumour vasculature, depending on the photosensitizer and other factors. A particularly interesting observation from PDT researchers is that it appears that PDT can have a significant effect on the immune system, and this can be either immunostimulatory or immunosuppressive. As noted by Gollnick and Brackett [125], pre-clinical and clinical studies have suggested that PDT creates a kind of systemic anti-tumour immunity, stimulating an adaptive immune system response. The working model is sketched in figure 25, following Castano *et al* [127], in which PDT is shown inducing both tumour apoptosis and necrosis, generating acute local inflammation and attracting dendritic cells (DCs) to phagocytose these necrotic cells. Cytokines released at the site stimulate DC maturation. These cells travel to lymph nodes where they present antigens to T lymphocytes, which following activation become effector T cells. Attracted by cytokines at

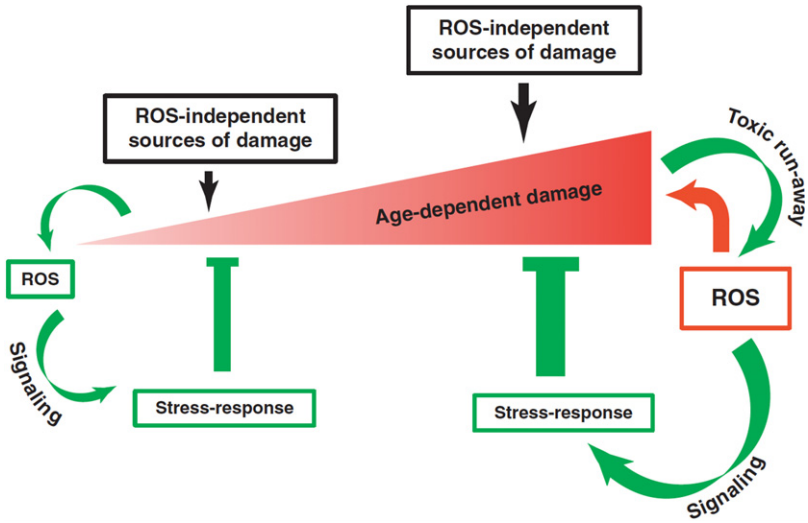

Figure 23. Following Hekimi *et al* [17]. Hypothesis is that ROS are generated as protective, stress response agents and when age-dependent damage is low (towards the left in the figure), ROS are handled by cellular detoxification mechanisms. As ageing progresses (move to the right), age-dependent damage increases and the attempt to respond to subsequent ROS-independent sources of damage by generating more ROS leads to increasing loss of control of ROS. As a result, ROS generated by the body in an attempt to reduce stress finally acts to amplify the damage as ROS can itself induce damage, triggering a toxic 'run-away' process. This would be expected to occur, however, only in the later stages of life, explaining the association of elevated ROS with age-related diseases and ageing itself.

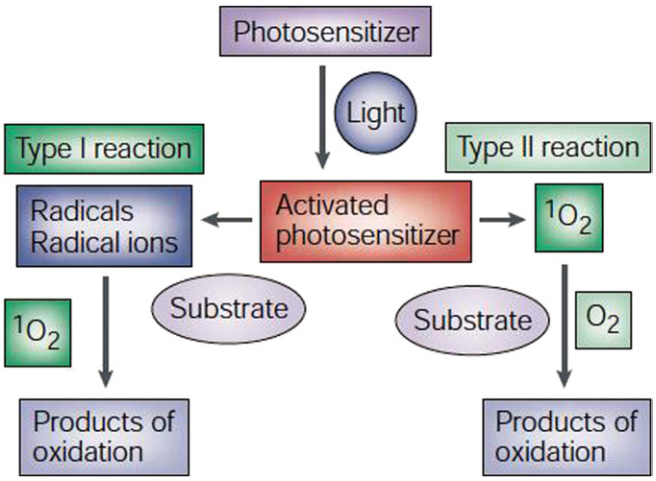

Figure 24. Following Dolmans *et al* [124]. Illustration of type I (left) and type II (right) reactions in PDT. Following absorption of light, photosensitizer may react directly with either cell membrane or other molecule, creating radical site. This radical site reacts with O_2 in solution to create $^1O_2$ in type I excitation. Alternatively, the excited photosensitizer may directly excite O_2 to form $^1O_2$ in a type II excitation reaction.

the tumour site, these effector T cells migrate to the tumour and kill tumour cells.

An obvious question for researchers using plasma-generated RONS to cause tumour cells to undergo apoptosis and perhaps necrosis [128] is whether something similar could happen to stimulate anti-tumour immunity in this case as well.

PDT has also been explored recently for use against localized infections [129]. Different photosensitizers need to be used against infectious agents, but only a few, relatively ineffective ones, have been tested due to limitations associated with toxicological and safety constraints. These authors point out that PDT has potentially many advantages against antibiotic-resistant stains of bacteria; it can act against a wider range of pathogens than antibiotics (fungi, bacteria, parasites and viruses), it might act against bacterial virulence factors (e.g. lipopolysaccharide from gram negative bacterial membranes) and it could show similar systemic immune system response as has been shown in some cases against tumours. The plasma medical researcher might point to very similar reasons that plasma therapy is attractive as well for these applications.

Photodynamic therapy has a number of factors in common with plasma-generated RONS therapy, as noted above, and this prompts the plasma medicine researcher to be especially interested in this technology. Techniques and approaches that have proven successful in PDT may also be useful to plasma medicine researchers. Similar comments can be made about potential problems. For example, Brown *et al* [130] noted that circa 2004, PDT had been in use for 25 years for oncology, with thousands of patients treated and considerable success in dermatological oncology and opthamology, but it had not really entered the mainstream in oncology overall. They suggest that the main reason for this relative lack of success is that PDT, while shown effective, was not obviously better than established treatments (e.g. radiation therapy, optimized for over 50 years) in the large clinical trials in which it was tested. This observation is no doubt relevant to plasma medicine researchers as plasma technology moves from lab to clinic—timescales associated with the introduction of new medical technologies are often measured in decades. Furthermore, efficacy, minimal side effects and even low cost may not be enough to rapidly displace existing therapies that are perceived to be about as good as the proposed new therapy. It will be important for the success of future plasma medicine technologies to identify and enhance the potential advantages of plasma approaches over existing, competing technologies like PDT. It is too early to say much about this, but plasma

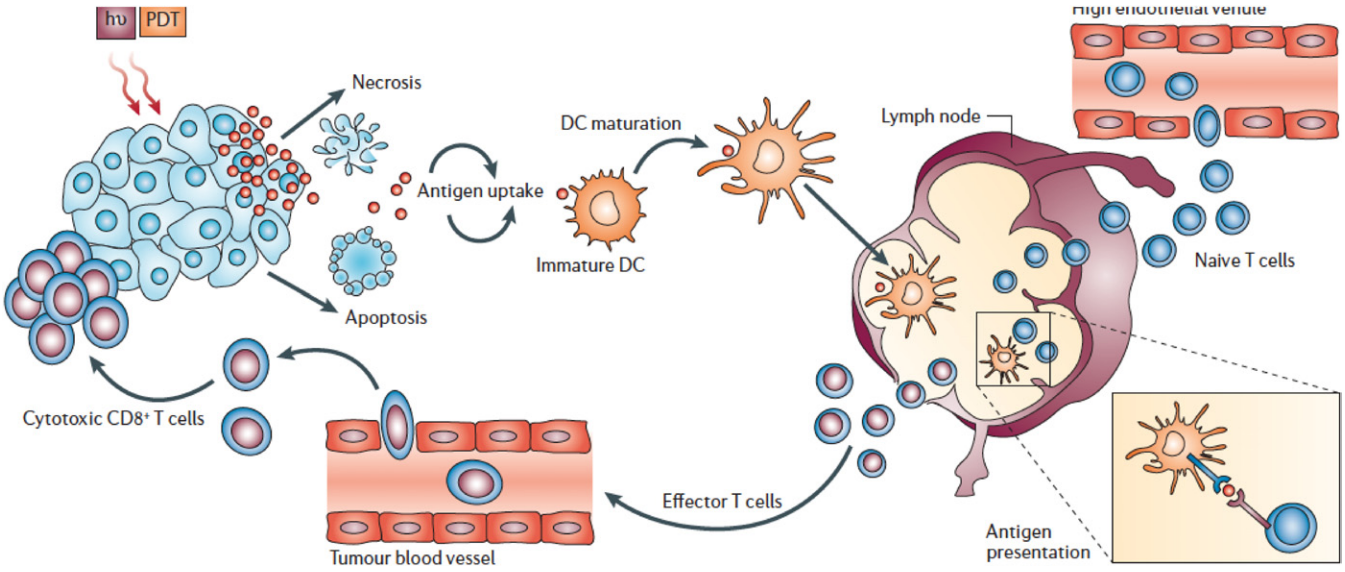

Figure 25. Following Castano *et al* [127]. PDT induces activation of antigen-specific T cells. PDT-generated $^1O_2$ creates both apoptotic and necrotic cell death. These cells are phagocytosed by DCs that have accumulated due to local inflammation. DCs mature after stimulation by cytokines, also released at inflammation site. These then move to lymph nodes where they present antigens to T lymphocytes. Activated T lymphocytes become effector T cells that are attracted to tumor by cytokines, resulting in their destruction.

appears to have the advantage of potentially creating many different kinds of reactive species than PDT. However, it seems that photosensitizer segregation into tissue to be treated (e.g. tumours) may give PDT an intrinsic selectivity advantage.

5.2.3. Ultrasound therapy. Ultrasound (>20 kHz) in liquid media is known to create reactive species during micro-bubble cavitation. In cavitation, gas bubbles grow to a certain point, then violently collapse. During this collapse, gas temperature can reach 5000 K and pressure may be as high as 800 atm. [131, 132]. In addition to light generation ('sonoluminescence'), various radicals are created, leading to 'sonochemistry.' There is a related approach to mechanically ablate material via ultrasound (e.g. kidney stones) known as 'lithotripsy' that does not rely on chemical effects. 'Sonodynamic therapy' is analogous to PDT in that ultrasound is combined with a 'sonosensitizing' compound that ideally localizes to a region such as a tumour. The combination of the sonochemically generated radicals and the sonosensitizer is thought to create a synergy that has therapeutic benefit. For example, Kondo *et al* [133] report that the combination of low-intensity pulsed ultrasound coupled with the chemotherapeutic agent doxorubicin (DOX) yielded a synergistic tumoricidal effect that they attributed primarily to radical formation.

5.2.4. Redox cancer chemotherapeutics. It has been known for over 40 years that many cancer chemotherapeutic drugs owe at least part of their effectiveness to the generation of ROS [134]. Table 2 lists a set of drugs commonly in use today that cause ROS stress either through pro-oxidant action or by blocking antioxidants [135]. The enhanced sensitivity of cancer cells to ROS stress is now thought to be due to the metabolic differences between normal and cancer cells. In 1933, Otto Warburg noticed that whereas non-malignant cells use O_2 to generate energy through the creation of adenosine triphosphate (ATP) via oxidative phosphorylation, cancer cells follow a radically different metabolic pathway and use oxidative glycolysis. As pointed out by many authors including López-Lázaro [136], one consequence of the metabolic differences (also known as the 'Warburg effect') is that tumors must generate and maintain relatively high levels of ROS, especially O_2^- and H_2O_2. In any case, the presence of higher than normal levels of ROS in most cancer cells is an established characteristic.

Table 2. Following Pelicano *et al* [144] Chemotherapeutic agents that cause cellular oxidative stress.

Mechanism	Agent
ROS generation	Arsenic trioxide
	Anthracyclines
	Bleomycin
	Bortezomib
	Cisplatin
	N-(4-hydroxyphenyl) retinamide
	Emodin
GSH depletion	Buthionine sulfoximime (γ-GCS inhibitor)
	Diethylmate
	Ascorbic acid
Inhibition of antioxidant enzyme	Merceptosuccinic acid (GPx)
	Aminotrizol (catalase)
	Ethacrynic acid, TLK199 (GST)
	2-Methoxyoestradiol (SOD)

The relation between ROS and antioxidants in cancer cells in illustrated in figure 26, following Cairns *et al* [137]. Relatively low levels of ROS lead to cell proliferation and survival pathways, but higher levels will lead to cell death. The metabolic changes and protein translation associated with cancer cells induce high ROS levels so they survive by expressing more antioxidants such as reduced glutathione (GSH) and thioredoxin (TRX) in the presence of NADPH, thus

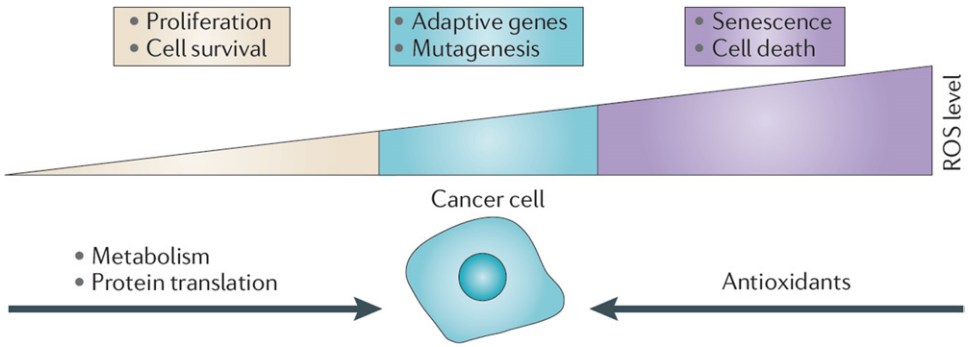

Figure 26. Following Cairns *et al* [137]. Low levels of ROS (left) are beneficial, leading to cell proliferation and survival pathways, but high levels (right) can lead to cell death. Cells use antioxidants to counter oxidative stress. In cancer cells, ROS levels are abnormally high due to metabolic differences and protein translation so they must express higher than normal levels of antioxidants, reducing ROS concentration to sustainable levels. But even this more moderate level of ROS concentration these may lead to additional mutagenesis.

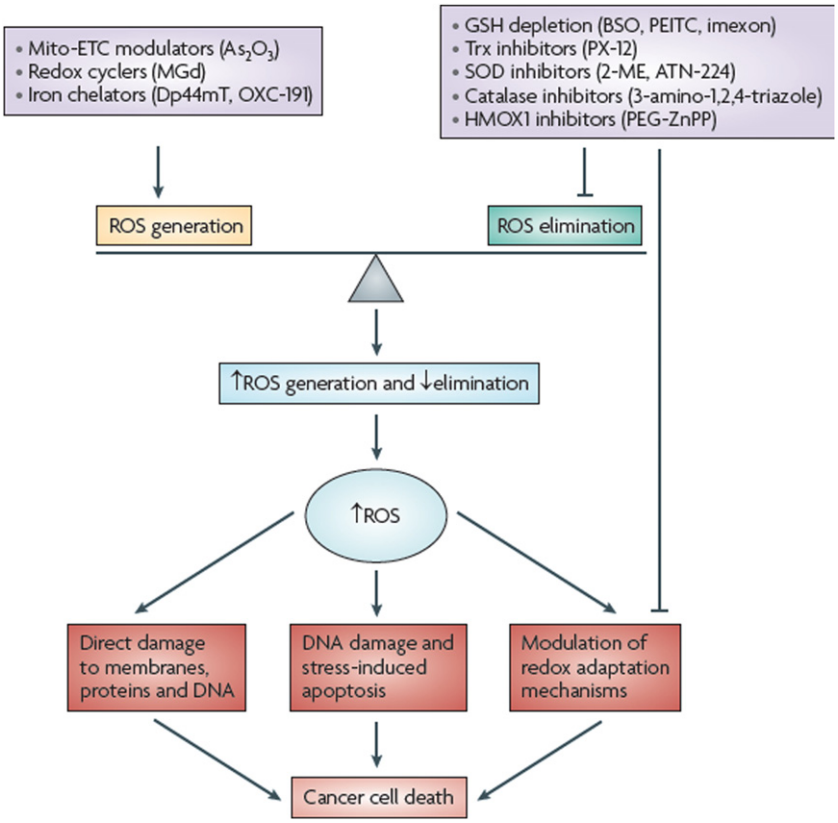

Figure 27. Following Trachootham *et al* [139]. There are two ways to increase ROS levels in cancer cells: increase rates of production (left path) and/or decrease rates of destruction (right path). The net effect of increasing ROS is to induce membrane, protein and DNA damage; induce apoptosis, and modulate redox adaptation mechanisms, all leading to cell death.

reducing ROS concentration to sustainable levels. In spite of this survival response, cancer cells can experience ROS-induced mutagenesis [137].

Over the last 25 years, the recognition that cancer is a disease of genetic mutation and that there are specific genes—named 'oncogenes'—that lead to cancer has suggested therapies targeting specific signalling molecules. However, the complexity of the cancer-related driver mutations and their multiple associated signalling pathways leading to tumor formation has raised questions about whether this strategy will ever be broadly successful [137]. One alternative might be to target aspects of the cancer cell metabolism, such as ROS.

As Wondrak [138] points out in a comprehensive recent review, 'redox chemotherapeutics' have emerged as a promising new direction for anti-cancer chemotherapeutics, especially for cancers with a few current options like melanoma and pancreatic cancer. Of course, it is recognized that ROS can be tumorigenic if present in lower concentrations, as noted above [109]. But the basic idea behind this therapy is to *increase* the levels of ROS in cancer cells to the point that they can no longer survive; antioxidant defense becomes overwhelmed and cells undergo apoptosis. Figure 27 (following Trachootham *et al* [139]), illustrates the basic idea. To increase ROS in cancer cells, the strategy is to increase

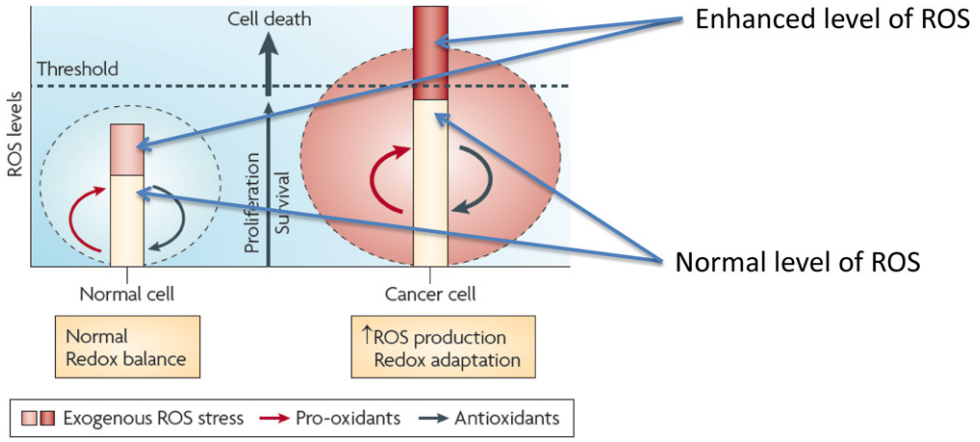

Figure 28. Following Trachootham *et al* [139]. This sketch illustrates the basic idea behind the use of redox chemotherapeutics. ROS levels in both normal and cancer cells are controlled by a balance between pro- and antioxidants, but both rates are higher in cancer cells, leading to higher baseline ROS concentration in cancer cells. An increase in ROS concentration will be induced by redox chemotherapy in both cell types, but in principle, only the cancer cell ROS level will exceed the 'death threshold,' thus providing a selective anti-cancer chemotherapy.

ROS-generation processes and/or decrease ROS elimination processes. The net result is that the increase in ROS causes the cancer cells to die.

Of course, this process needs to be as selective as possible so normal, non-cancer cells are not affected or at least not too much. The proposed mechanism behind this clinically observed selectivity is shown in figure 28 (also following Trachootham *et al*, [139]). Cellular ROS levels are controlled at steady state by pro- and antioxidants. However, both creation and loss are higher in cancer cells, leading to higher baseline ROS concentration in cancer cells. Redox-inducing chemotherapy will raise ROS concentration in both cell types, but in normal cells, the antioxidants in reserve will keep the ROS concentration from reaching the apoptosis-inducing threshold. Cancer cells, with their higher basal ROS concentration, have no excess antioxidant capacity, and are forced into apoptosis. This strategy has received considerable attention in the last several years and has led to numerous clinical trials (see, e.g., [108, 134, 138, 139–145]).

Chiurchiu and Maccarrone [108] write, in referring to the aforementioned review by Wondrak [138]:

> The impressive number of ongoing clinical trials that examine therapeutic performance of these novel redox drugs in cancer patients demonstrates that redox chemotherapy has already made the crucial transition from bench to bedside.

It is worth special note that Fruehauf and Trapp [143] report that several redox chemotherapies have proven promising clinically against melanoma: chemotherapeutic drugs ATN-224 and disulfiram were shown clinically to block ROS scavenging and ROS-generating agent elesclomol, along with paclitaxel. This drug protocol led to improved patient survival in a phase II clinical trial against melanoma. One of the first examples of successful *in vitro* anti-cancer results using air plasma was reported in 2010 by Sensenig *et al* [128]. Their *in vitro* target cancer cell was melanoma, and furthermore, they showed that ROS scavengers negated the tumoricidal effects of plasma treatment. These authors concluded that ROS are primarily responsible for plasma-aided cancer treatment.

Similar conclusions about the importance of ROS in plasma-aided cancer treatment were drawn recently by van Damme *et al* [146]. These authors, following up on the first published reports of *in vivo* plasma cancer therapy using a mouse model and human glioma xenograft [147], extended this study to a combined *in vitro* and *in vivo* study of human glioblastoma U87MG and colorectal cancer HCT-116 [146]. They inferred from their studies that the plasma-created ROS were the key anti-tumor chemical agents.

5.2.5. RNS in cancer therapy. Space limitations will allow only a short summary of this important and emerging topic. Hirst and Robson [148] focus on nitric oxide and the role of nitrosative stress in cancer therapy. In many ways, this mirrors the discussion about ROS and cancer. Both species are known to be related to tumorigenesis at lower concentrations, but can be effectively anti-cancer at higher concentrations. NO and NO-related donor drugs have been shown to be excellent sensitizers to either radiation therapy or chemotherapy. In particular, Hirst and Robson [149] point out that this approach has been successful sensitizing (i.e. increasing the effectiveness of, and/or reducing chemoresistance for) the ROS-generating chemotherapy cisplatin.

Other papers that review this topic include Thatcher [150], Sullivan and Graham [151], Jamier *et al* [152], Singh and Gupta [153], Coulter *et al* [154], Hirst and Robinson [149] and Lechner *et al* [155].

5.2.6. Redox drugs: anticancer and antimicrobial effects. A final note in this discussion of cancer therapies is the connection between antibacterial agents and cancer chemotherapies. There are a number of compounds that have proven effective as antibiotics, anti-fungal or anti-parasitical compounds that also work against cancer. The idea that RONS-generating drugs or ROS-generating light sources (like PDT, noted above) should kill microbes of course is also consistent with what is well known from the immune system. Phagocytes use the same chemistry to kill microbes and they are also known

to be anti-tumoral as well. This connection has been noticed for some time, although it has not received a lot of attention.

A paper published in 1997 by Gutteridge *et al* [156] summarized the idea, and they use the term 'phagomimetic' to describe the connection. Quoting from the abstract of this paper entitled '*Phagomimetic Action of Antimicrobial Agents*:'

> A wide variety of extracted and synthesized drug molecules have electron transfer capabilities which allow them to generate ROS. In particular, many antibiotics that kill or inhibit bacteria, yeasts and cancer cells readily transfer electrons to oxygen, making superoxide and hydrogen peroxide in the process. When suitable redox active forms of iron are available, Fenton chemistry occurs generating the highly damaging hydroxyl radical. This type of chemistry is very similar to that which evolved within phagocytic cells as part of their microbial killing armoury.

These authors note the immunomodulatory effects of these agents, and also note the history of this general concept (e.g. Crawford *et al* [157]).

Although not used as a medicinal, the effects of the herbicide paraquat, noted below in the section on plants, acts in a similar way. Paraquat is a redox cycling compound, taking an electron to form an anion, then transferring this electron to O_2 to create O_2^-, allowing the anion to form again in a repetitive cycle [14].

One of the most recent examples of this connection are the antimalarial drugs artemisinin and its synthetic organic endoperoxide variants, as noted below. (Some of these variants, incidentally, are so-called 'ozonides,' since they are made by reacting organic precursors with ozone.) Wondrak [138] reports that the main mechanism of various artemisinin compounds is the induction of cellular oxidative stress, mitochondrial dysfunction, followed by mitochondrial-triggered apoptosis. It is not only consistent with the theme of this review that RONS that are active in one area are active in another area of therapy, but it really is a necessary condition for the principle of RONS-active therapies. These compounds are discussed further in the section on anti-parasiticals.

5.3. Infectious disease therapy

5.3.1. Antibiotics. In 2007, an influential and highly cited paper on the mechanisms responsible for antibiotic lethality was published by Collins *et al* [158]. In this publication, the authors showed evidence that for all three major classes of antibiotics, (i.e. attacking either DNA, cell membranes or ribosomes) the bactericidal action was ultimately connected with the creation of ROS. Specifically, they showed that at the end of a long sequence of reactions involving bacterial metabolism, the final result was creation of excess O_2^- followed by formation of the highly reactive hydroxyl radical through the iron-catalyzed Fenton reaction. (see figure 8(*b*)) The Fenton reaction (converting H_2O_2 into OH radical via Fe^{2+} catalytic oxidation) coupled with O_2^- induced, Haber–Weiss reduction of Fe^{3+} to Fe^{2+} completes the redox cycle. This is the same cycle that was proposed by Kell as the postulated operative mechanism in many other ROS-induced degenerative diseases [116, 117]. In the case of the antibiotics, the detailed differences between prokaryote and eukaryote cellular biochemistry are responsible for the fact that the antibiotics are (generally) harmless to human cells but result in lethal radical creation in susceptible bacteria.

The OH radical created by these catalytic cycles was presumed to be responsible for the damage that led to bacterial cell death since there are no known enzymatic pathways to detoxify the very rapidly reacting radical. While generally accepted as an exciting and novel insight into the mechanisms of antibiotics, the paper's conclusions regarding oxidative stress as the sole mechanism of antibiotic action were thought to be at least somewhat over-broad. For example, Hassett and Imlay argued that many antibiotics worked in oxygen-poor regions such as within biofilms and were thus not likely to exclusively exploit this oxidative stress mechanism [159]. In addition, many pathogens have evolved to resist RONS generated by the human immune system, but remain susceptible to antibiotics [80]. It should also be pointed out that similar, but not identical, ideas predated this paper [156, 157].

Collins *et al* followed up this highly cited paper in 2009 and 2010 with additional aspects of the ROS-antibiotic connection [160, 161]. The 2009 paper addressed the question of antibiotic resistance and its relation to the ROS bactericidal mechanism. They suggested that the ROS connection might be exploited to design novel strategies to extend existing antibiotics and confound bacterial mutational and horizontal gene transfer, thus countering antibiotic resistance. In the 2010 paper [161], evidence was presented that sub-lethal doses of bactericidal antibiotics cause mutagenesis through the formation and action of ROS, perhaps due in part to antibiotic-induced oxidative stress response. The ROS-induced mutagenesis in turn leads to multi-drug antibiotic resistance.

Park *et al* [162] reported that the well-known antibacterial element silver is associated with the formation of O_2^-. These researchers found that silver ion antibacterial effectiveness was enhanced under aerobic conditions and that the established silver ion–thiol interaction mechanism was related closely to the ROS mechanism in a synergistic manner.

5.3.2. Anti-parasitical agents. It has been known for some decades that parasites are vulnerable to drugs that act through the creation of free radicals [163, 164]. In most cases, these radicals either directly or indirectly involve the oxygen- and nitrogen-containing radical species discussed in this paper. Nitric oxide (NO) has been particularly identified as an important anti-parasitical agent, and will be discussed further below [165, 166]. In order to abbreviate the discussion here, the focus is mainly on chagas disease (CD), leshmaniasis and malaria, but the issue spans many more parasite infections and associated ailments.

Chagas disease (CD) is a major parasitic disease in Latin America and elsewhere. The causative parasite is *Trypanosoma cruzi*, a hemoflagellate protozoan [167]. The two currently available drugs are nitroaromatic compounds, Nifurtimox (Nfx) and Benznidazole (Bnz). Various drugs

have been proposed over the years, and many of these are thought to act by the generation of oxidative stress through creation of O-containing radicals. Some examples include quinone and quinone immines, nitrofurans and nitriimidizoles, triarylamines and aminoquinolines, among others [168]. Both Nfx and Bnz are thought to involve radical generation, but the main trypanicidal activity of Nfx has been identified as ROS generation [169]. Efforts to develop analogous drugs with perhaps fewer undesirable side effects, but still involving radical generation and oxidative stress, have been made. Examples include etanidazole [170] and molecular hybrids of hydrazone and benzofuroxans [167]. In all of these cases, it appears that the trypanicidal effect is related to the generation of oxidative stress. Interestingly, related compounds have shown promise as radiation sensitizers for hypoxic cancerous tumors [171, 172].

Leishmaniasis manifests itself in either its visceral or tegumentary (cutaneous and mucosal) forms, and is due to infection by the protozoan parasite *Leishmania* from sand fly bites. Some of the drugs either in use or being proposed today appear to act, at least in part, through imposition of oxidative stress via ROS. One such compound, tafenoquine (an 8-aminoquinoline being tested for its antimalarial action), appears to affect Leishmania mitochondria, leading to an 'apoptosis-like death process.' [173] Kumar *et al* [174] report that nelfinavir, an HIV-1 protease inhibitor, generates oxidative stress in Leishmania amastigotes, leading to apoptosis. This is thought to be especially significant given the common coupling between HIV/AIDS and Leishmaniasis. Finally, it is worth noting that Fridman *et al* [2] have shown that air plasma treatment of cutaneous leishmania promastigotes leads to growth inhibition.

Malaria is arguably one of the most important infectious diseases in the world. The disease is caused by a protozoan parasite (genus *Plasmodium*) infecting red blood cells (or 'erythrocytes') following the bite of an infected female mosquito (genus *Anopheles*). ROS and oxidative stress are known to be important in malaria [175, 176]. The pro-oxidant nature of many antimalarial drugs is widely, although not exclusively, thought to be the mechanism of these antimalarial agents. And malaria-induced oxidative stress appears to be at the heart of many of the most serious, even fatal, consequence of the disease. The latter include severe anemia and respiratory problems in cerebral malaria affecting mainly African children; and kidney failure and respiratory distress/lung pathology in infected Asian adults [176]. The mechanisms involved in chemotherapeutic antimalarials have been controversial, especially for artemisinin and related endoperoxides (discussed below) [177]. Interestingly, the recent review by Schlitzer [178] did *not* emphasize the role of oxidative stress in the action of many antimalarial drugs.

However, most authors appear to acknowledge that redox chemistry is central to the effectiveness of many antimalarials [175, 176, 179]. Becker *et al* [176] list, among others, various aminoquinolines as well as artemisinin and its various derivatives, and methylene blue, as acting somehow through an oxidative stress mechanism. The most common mechanistic arguments centre around the interactions with either unliganded Fe^{2+} or heme, or both (180–182]. Wang *et al* [182] argue that the antimalarial mechanism of artemisinin, and perhaps the other peroxide-structured antimalarials, work selectively by being activated only in the malaria (and yeast) mitochondrial electron transport chain, then creating ROS locally to disrupt the mitochondria function.

An intriguing development in antimalarial artemisinin-related drugs is the announcement of successful clinical trials of novel synthetic ozonides [183]. Synthetic ozonides contain a similar structure as artemisinin and its derivatives, namely the endoperoxide bridge mentioned above. These compounds are made by exposing promising starting compounds with alkene structures to ozone (O_3), but development of effective drugs requires careful consideration of many factors in addition to its pharmacological effectiveness, including stability, solubility and lifetime [184]. It is tempting to suggest that plasma-generated RONS (including O_3) could create similar structures when exposed to biological fluids, or perhaps to selected precursor molecules, leading to plasma-generated drugs, but this has not been demonstrated to date.

As noted in the previous section on cancer therapies, the recent discovery that various artemisinin derivatives can be tumoricidal is intriguing from the point of view of this paper. Stockwin *et al* [185] report that artemisinin dimer results in ROS generation, the involvement of iron and/or heme, DNA damage and ultimately in apoptosis. In their recent review, Nakase *et al* [186] report that artemisinin derivatives have proven effective in inhibiting cancer cell growth for many different types of cancer cells, both *in vitro* and *in vivo*. These authors suggest that cancer cell specificity of artemisinin is due to its reaction with iron-containing molecules (and Fenton chemistry) in or on the cancer cells, where the resulting catalytic creation of ROS ultimately leads to apoptosis. In some cases, the artemisinin derivatives proved to be anti-angiogenic as well [186].

5.4. Nitric oxide (NO)

Of all the RONS, it is undoubtedly nitric oxide (NO) that has received the most attention. NO is truly an enormously important molecule biochemically, both for its role in normo- and pathophysiology and for its importance, directly or indirectly, in a suite of therapies. By some estimates, on the order of 100 000 scientific papers have been published on this compound and its biomedical actions [187]. Obviously, the aforementioned note about the brevity of this paper's coverage is amplified many times for the summary of this centrally important molecule.

Exploration of the use of NO as an adjuvant to minimize chemo- and radiation-therapy resistance in cancer therapy was noted above. But both gaseous nitric oxide and various NO donor drugs are in use for a variety of other disorders and afflictions.

5.4.1. Gaseous NO. NO is one of the few RONS to be used directly in therapy. Although it is a radical with an unpaired electron, it can be stored as a fairly stable gas for a reasonably

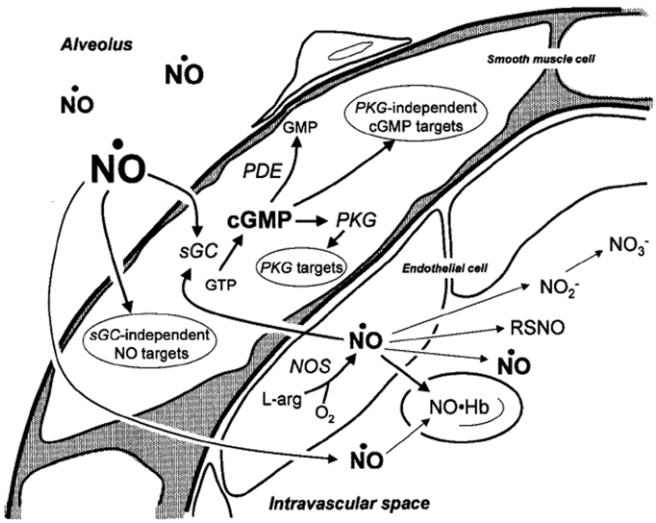

Figure 29. Following Ichinose *et al* [188]. Sketch illustrating NO signalling pathways in the lung. NO is shown, among other roles, interacting to stimulate the formation of cGMP. PKG indicates cGMP-dependent protein kinases; NOS is NOS; L-arg is L-arginine; sGC is soluble guanulate cyclases; and RSNO are S-nitrosothiols.

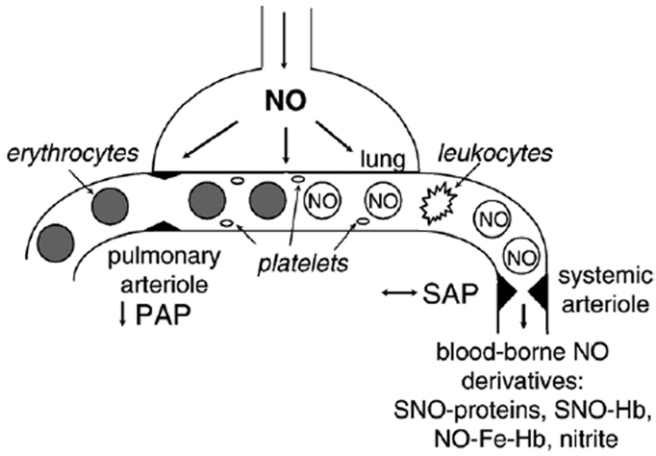

Figure 30. Following Bloch *et al* [189]. Inhaled NO is a selective pulmonary vasodilator with actions on the systemic vasculature. A schematic of an alveolar-capillary unit is shown highlighting NO acting to dilate pulmonary arterioles and reduce pulmonary artery pressure (PAP). Inhaled NO shows some systemic effects due to actions on circulating cells. For definitions and further information, see original reference.

long period of time and can be purchased from gas suppliers. Figure 29, following Ichinose *et al* [188], illustrates some of the signalling pathways of NO in the lungs. Figure 30, following Bloch *et al* [189], demonstrates one hypothesis about how NO enters the systemic vasculature. The most common current (FDA-approved) therapeutic applications are for pulmonary hypertension and hypoxemia in adults and newborn infants. In addition, gaseous NO had been explored for use with various cardiovascular, cutaneous vasculature and vascular smooth muscle disorders [190]. Inhaled NO is generally applied at no higher than 80 ppm [191]. Higher concentrations are thought to lead to formation of NO_2, which is known to be a significant irritant in the lungs.

Gaseous NO has also been used successfully on wounds to disinfect and speed healing. Miller *et al* [192] report a single case study of the use of gaseous NO to treat a chronic non-healing leg ulcer. In this study, 200 ppm NO was applied over periods of 8 h for 14 days with little discomfort or side effects. In addition, it is currently in clinical trials for treating non-healing wounds, various fungal and parasitic infections, sinusitis, cerebral malaria and cystic fibrosis [193].

The relevance of NO to this paper should be clear: it is a reactive radical and is itself created in significant quantities in air plasmas and rare gas/air jet plasmas (e.g. plasma needle). From the point of view of plasma biomedicine there have already been several reports in the literature describing the use of gas plasma systems to create and deliver mostly NO. For example, Shekhter *et al* [194] and Dobrynin *et al* [195] describe gas plasma methods to generate gaseous NO for various biomedical applications.

5.4.2. NO donor drugs and NO delivery systems. Nitric oxide donor drugs and other NO delivery systems have received considerable attention. Recent reviews include Napoli and Ignaro [196], and Miller and Megson, [197]. Organic nitrates such as glyceryl trinitrate (or 'trinitroglycerin') and amyl nitrite (i.e. trimethyl butyl nitrite) have long been used for chest pain (angina). A number of other drug candidates have been proposed, but Miller and Megson [197] point out that surprisingly few clinically proven drugs have emerged from this group. As noted above, Hirst and Robson [149] review the use of gaseous NO various NO donor drugs to increase tumor sensitization for chemotherapy and radiation therapy for cancer treatments. Hybrid drugs such as NO-releasing aspirins have been tested [196].

Various novel NO delivery techniques have been suggested, such as microporous zeolites, and these are thought to be promising for applications such as antimicrobial coatings for catheters and wound dressings [197]. Nano-structured particles such as dendrimers [198], nano-particles [199] or block co-polymer stabilized nano-particles [200] have all been reported, among other schemes, to deliver gaseous NO. Finally, as noted in the section below, inorganic nitrites can also act as NO donors, especially in an acidic medium.

5.5. Inorganic nitrites: the nitrate, nitrite, nitric oxide pathway

As noted in the section of RONS and cellular homeostasis, in the last decade or so, there has been an extensive literature on the so-called nitrate–nitrite–nitric oxide pathway. (e.g. [201–207]) The nitrite anion (NO_2^-) is now thought to be the major intravascular and tissue storage form of NO [201]. This is especially relevant to plasma biomedicine because of the fact that antimicrobial acidified aqueous nitrite and nitrate anions are known to result from exposure of water to low-temperature air plasmas, as noted above in the section on plasma sources [49, 50, 208]. As Lundberg *et al* [97] point out, there are various ways for NO to form from the reduction of NO_2^- in the body, including haemoglobin, myoglobin, ascorbate, polyphenols, xanthine oxidoreductase

and protons (i.e. acidic environments). NO can also be formed from UVA (315–400 nm) photolysis of NO_2^- in aqueous solution [190].

The history of nitrate and nitrite is fascinating, as highlighted by Gladwin *et al* [201]. As early as 5000 years ago, there is evidence that nitrate salt was used for food preservation; this use persists today and includes nitrite as well. Evidence was recently uncovered concerning the medicinal use of nitrate/nitrite for cardiovascular disorders in 8th century China. It was not until the late 1980s, however, that researchers realized that nitrate and nitrite were coupled with nitric oxide and that these compounds have centrally important biological roles [201].

As noted above, for about 50 years, there has been concern about the harmful (carcinogenic) effects of dietary nitrate (e.g. sodium nitrite, $NaNO_2$, used for meat preservation among other things), due to its potential creation of carcinogenic N-nitrosamines. This has mostly been disproved—epidemiology studies have not shown a convincing association. One study even showed that acidified nitrite at concentrations and pH found in the stomach or urine will *inhibit* the growth of bladder cancer cells. It is now known that when nitrate is reduced to nitrite and then to nitric oxide in the body, this NO plays a multitude of important roles. On the other hand, as pointed out by Gilchrist and Benjamin [209], there is a possibility that some people may be more susceptible to gastric cancer than the general population and this group may be advised to minimize nitrate intake.

The other concern regarding nitrate consumption is the possibility of infant methemoglobinemia ('blue baby' syndrome) associated with well water containing high concentrations of nitrate. This concern has now been largely shown to be unimportant as well, although many governments continue to limit levels of nitrate in drinking water [209].

In recent years, there has been an explosion of interest in the nitrite anion (NO_2^-) as a therapeutic drug. (e.g. [97, 210, 211]) It turns out that nitrite leads to significant vasodilation, possibly through the release of NO under hypoxic and acidic conditions. When blood flow has been cut off from some region of the body ('ischemia') and then blood flow returns ('reperfusion'), this can lead to serious damage, referred to as 'ischemia/reperfusion' (I/R) injury. Nitrite has been shown to be effective in reducing I/R injury. Kevel and Lefer [207] report that in 2010 there were eight separate nitrite drug clinical trials underway in the US for various cardio-protective and vasculo-protective applications. Nitrite-containing drugs are aimed at treating myocardial infarction (heart attack), stroke, solid organ transplantation and sickle cell disease.

A standard way to treat wounds is through various topical NO donors. One common method is the application of an acidified nitrite cream, although there has been discussion about it causing skin irritation/inflammation. The acidified cream is thought to create a steady supply of NO that acts to kill bacteria and skin fungus and to promote wound healing [212, 213]. Butler and Feelisch [214] summarize in a recent review the history and current uses of inorganic nitrate and nitrite.

5.6. Ultraviolet (UV) light

UV radiation is the part of the electromagnetic spectrum with wavelengths between 200 and 400 nm. Below 200 nm, the term 'vacuum ultraviolet' is used since atmospheric air tends to absorb the radiation at these wavelengths relatively rapidly. UV is divided into UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). UVA is sometimes divided into UVA1 (340–400 nm) and UVA2 (320–340 nm). Various therapies using UV light work, at least in part, via the creation of ROS, especially for dermatology, as outlined below.

In addition to therapeutic uses, UV light has of course long been used to disinfect water and surfaces contaminants. Recently, interest has increased in light emitting diodes (LEDs) for this application [215]. UV radiation is known to generate RONS by photolysis of precursors such as H_2O, O_3, H_2O_2, HNO_2 and HNO_3 generating a range of RONS. These include $^1O_2$, O_2^-, OH, NO and NO_2 [216].

It is well known that excessive cutaneous UV radiation (e.g. from the sun) can lead to tissue damage, immunological disruption and even carcinogenesis [217]. The presence of radicals in the skin from both endogenous and exogenous sources is well known [218]. In some ways, the damage-therapy dichotomy observed with UV is similar to the pattern for RONS, since therapeutic effects are also observed; some of these beneficial applications are summarized here. The creation of RONS has not necessarily been established in all cases of UV therapeutic applications, but the fact that these species are generally created when interacting with water and other common compounds, as noted above, suggests that UV-related therapies are closely related to RONS.

The use of UV radiation for skin disease therapy began in the early 20th century [219]. In 1903, the Danish physician Finson won the Nobel Prize for medicine or physiology for his invention of a technique using UV light to cure skin tuberculosis. In 2005, Moller *et al* concluded that the acting therapeutic mechanism in Finson's technique was UV creation of singlet O_2 ($^1O_2$) through excitation of endogenous porphyrin structures within the bacteria [219]. In addition to endogenous porphyrins, a common class of exogenous photosensitizer is the psoralens. The use of photosensitizers in combination with external light is of course very similar to visible light-stimulated PDT, discussed above in greater detail. Recent results have shown that infrared radiation can also generate radicals in skin, as reported by Darvin *et al* [220].

In current dermatological UV therapy, there are three major wavelength ranges in use: narrow band UVB (311–312 nm), UVA and UVA1 (340–400 nm). In the latter category, Godar and Lucas report that the major applications are for various inflammatory skin diseases like atopic dermatitis and psoriasis, among several others [221]. These authors point out that about 30% of UVA1 radiation penetrates into the dermis, where T and B lymphocytes circulate through capillaries. The radiation creates $^1O_2$ that in turn induces T cell death by prompt apoptosis.

Morita points out that UVA application in the presence of the psoralen class of photosensitizer (termed psoralen plus UVA or 'PUVA') has been used for various skin diseases for

over 30 years, but that more recently narrow band UVB and UVA1 have gained greater acceptance [222].

There is epidemiological evidence, reported by Ponsonby *et al* that UVB radiation may play a protective role in suppressing several autoimmune diseases: MS, insulin-dependent diabetes mellitus, and rheumatoid arthritis [223]. These authors note that the cause may be increases in serum vitamin D levels. Both broadband and narrow band (310–313 nm) UVB radiation have been used to treat psoriasis, in some cases since the 1920s [224].

Ahmad [225] proposed using a combination of H_2O_2 (as a sensitizer) and UVA from the sun as a way to inexpensively treat a variety of skin diseases, especially leprosy. He demonstrated a synergistic effect of H_2O_2 with UVA and emphasized the potential value of this therapy in low resource countries. Thai *et al* demonstrated success in using UVC to treat chronic wounds infected with resistant bacteria (MRSA) [226]. Johnson *et al* reported that UV-generated ozone effectively inactivated pathogenic prions [227].

5.7. Ozone

Ozone (O_3) has a curious and controversial history as a medically useful gas, the details of which are well beyond the scope of this paper. Of course, ozone has been well established as a commercially useful way to disinfect water supplies since the late 19th century, using mainly DBD technology invented by Siemens in 1857 [228]. But its use as a medical agent has been much more controversial. In 1916 and 1917, and again in 1940, reports appeared by military battlefield doctors touting the efficacy of ozone therapy, either as a direct gas treatment or after dissolving in normal saline, for wound treatment [229–232]. Apparently, however, little was published in the English language scientific or medical literature until relatively recently. Bocci *et al* [233] provide a much more complete history. In 1993 an unauthored paper entitled '*Questionable methods of cancer management: hydrogen peroxide and other 'hyperoxygenation' therapies*,' was published in the American Cancer Society's journal CA: A Cancer Journal for Clinicians [234]. This paper, still cited by the Society, recommends against the use of ozone and other 'hyperoxygenation' therapies such as H_2O_2 for cancer treatment, although it concludes with the observation:

> This recommendation, however, is not intended to preclude the use of oxygen-rich compounds or hyperbaric oxygen therapy in situations for which efficacy has been demonstrated. Nor is it meant to discourage responsible study of these methods by the scientific community [234].

A strong proponent for the proper medical use of ozone is V A Bocci. He notes the potentially toxic properties of ozone can be controlled if properly administered, leading to demonstrable therapeutic value for numerous disorders. Several of his recent reviews [233, 235] and a book [236] have highlighted some important aspects of the medical use of ozone, including its possible or likely role as a 'hormetic' agent, noted above.

Based on his own and other practitioners' clinical results, but lacking large-scale clinical trials, Bocci has argued emphatically and extensively that ozone, properly used, can be effective in treating many disorders. These include peripheral obstructive arterial diseases, age-related macular degeneration, chronic infectious diseases, pulmonary diseases, orthopedics and dentistry [233, 235].

5.8. Low-level laser (light) therapy

Another emerging but still apparently controversial therapy involves the use of low-level (or low intensity) light therapy (LLLT) for wound healing. Tafur and Mills [237] report that both visible and near-visible low-intensity light affect cells through existing cellular redox mechanisms. Hamblin and co-workers [238] conclude that LLLT in the visible and near-infrared induces the activation of NF-κB and possibly other redox-sensitive transcription factors. These authors suggest that LLLT is pro-oxidant in the short term but antioxidant in the long term. Lubart and co-workers stress the important mechanistic role played by ROS creation in LLL effects in wound sterilization and healing [239, 240].

6. Plant biology and RONS

6.1. Introduction

The roles of RONS and redox chemistry in plant biology are no less important than they are for animals or bacteria. Although there have not been many attempts to date to use plasma sources of RONS to affect plants, there have been a few. For example, several studies have examined using plasma for seed germination [241–244] or simply to measure the effects of plasma on plants [245]. The evidence to date suggests that some effects can be seen, and the fact that plasmas operated in air often yield NO, a known factor in seed germination, is probably related [243]. One can imagine many possible future applications, including the use of plasma to protect plants from fungus or other microbial or even parasite attack, but this will require much additional work. In the meantime, it will be helpful to investigate more deeply the roles played by RONS in conventional plant physiology and disease.

A recent comprehensive review by Foyer and Noctor [14] presents the control of redox reactions as being at the heart of plant physiology. They point out that plants began to use the energy associated with the 0.815 mV redox couple between O_2 and H_2O about 3 billion years ago, and this has shaped plant biochemistry in profound ways. These authors stress the role that ROS play in the regulation of gene expression. Moller *et al* [246] describe many reactions between RONS and plant cell components, including proteins, lipids and carbohydrates. In a recent review, Hadacek *et al* [247] propose that the purpose of secondary plant metabolites, whose function beyond the general notion of 'plant defence' is still a point of controversy in plant biology, is intimately connected with RONS chemistry. These authors hypothesize that secondary metabolites exist to serve primarily as RONS reaction partners and that these reactions are central to the hormetic effects of plant secondary metabolites, such as phenols, terpenoids and alkaloids. A

Table 3. Following Foyer and Nocter [14]. Summary of plant-derived ROS under physiological conditions. Protonated forms of hydrogen peroxide and superoxide exist as well. The asterisks denote relative reactivity. 'Radical' refers to species with an unpaired electron and 'ion' of course denotes a charged species.

Name	Symbol	Radical	Ion	ROS	Primary sources in plants
Triplet oxygen	$^3O_2$	Yes	No	No	Photosystem II
Singlet oxygen***	$^1O_2$	No	No	Yes	Photodynamic transfer (excited chlorophylls, etc)
Superoxide**	O_2^-	Yes	Yes	Yes	Electron transport chains, oxidases
Hydrogen peroxide*	H_2O_2	No	No	Yes	Reduction/disumutation of superoxide, oxidases
Hydroxyl radical****	OH·	Yes	No	Yes	Reductive cleavage of H_2O_2
Water	H_2O	No	No	No	Absorption, various reactions
Hydroxide	OH^-	No	Yes	No	Various reactions, ionization of water

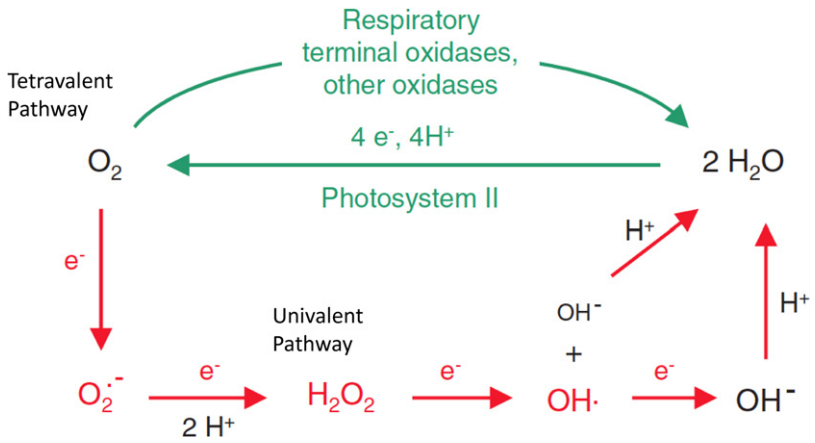

Figure 31. Following Foyer and Nocter [14]. Two main paths of oxygen reduction in plants. Respiratory and other oxidases transform O_2 to water in the upper tetravalent pathway along with corresponding tetravalent oxidation of water via photosystem II. The univalent pathway (below) shows how individual electron additions yield various ROS: O_2^-, H_2O_2 and OH. Not shown are photosynthetic pathways leading to formation of $^1O_2$ (see table 3).

subset of plant secondary metabolites, namely the flavonoids, have been used for thousands of years in traditional Eastern medicine and are beginning to be explored for their therapeutic benefit in modern medicine [248]. Among their known effects is their effectiveness as antioxidants. Jacob *et al* discuss the use of redox active secondary metabolites, especially those that induce pro-oxidant response, and not only from plants but also from bacteria and fungi [18]. These authors note the potential role of the compounds in cancer therapy and to counter autoinflammatory diseases.

Plants can be damaged or killed if redox reactions are not controlled. For example, the powerful herbicide paraquat (aka methyl viologen) is known to act through so-called redox cycling in which the iron–sulfur groups of photosystem I provides electrons to paraquat, creating the anion. This donates an electron readily to O_2, creating O_2^- superoxide, resulting in local plant cell death if the antioxidant enzymes are unable to counter the effect [14].

Table 3 lists the primary oxygen-containing small molecules, including ROS, in plants and the location of their creation [14]. Figure 31 from Foyer and Noctor shows the two paths between O_2 and H_2O: in what is termed photosystem II of the photosynthetic electron transport (PET) chain, H_2O is oxidized via light and enzymatic processes to O_2; the reverse reaction, part of the respiratory electron transport (RET) chain, reduces O_2 to form H_2O. These two processes are 'tetravalent' pathways, simultaneously involving all four of the electrons involved, so that this pathway does not yield any ROS. By contrast, the parallel 'univalent' pathway from O_2 to H_2O creates ROS along the way, including O_2^-, H_2O_2 and OH. Singlet O_2 ($^1O_2$) is mostly created via photoexcitation in green plants in the chloroplast, the location of photosynthesis. As in all other aerobic organisms, in order to control ROS there must be antioxidants, the most important of which in plants are NADPH, glutathione and ascorbate.

Foyer and Noctor [14] focus on ROS, but RNS are very important in plants as well. Figure 32(*a*) illustrates the major ways that NO is formed in plants; figure 32(*b*) shows various NO-based signalling pathways in plants [249]. Moroz [250] discusses the relations between NO signalling in prokaryotes, plants, fungi and invertebrate animals, showing the strong similarities between these different groups and as a function of evolutionary time. Gaupels and Kuruthukulangarakoola [251] summarize current knowledge about NO signalling in plant defence against pathogens, stressing that there are still big gaps in understanding.

ROS are created more or less continuously in plant respiratory and photosynthetic pathways, but are generated in a 'burst' mode in response to specific events associated with growth and in response to environmental stress challenges, including pathogen attack or salt stress.

6.2. Activation of plant defenses

Plants are of course subject to a variety of attacks from bacteria, fungi, viruses, insects and so forth. Their response to these

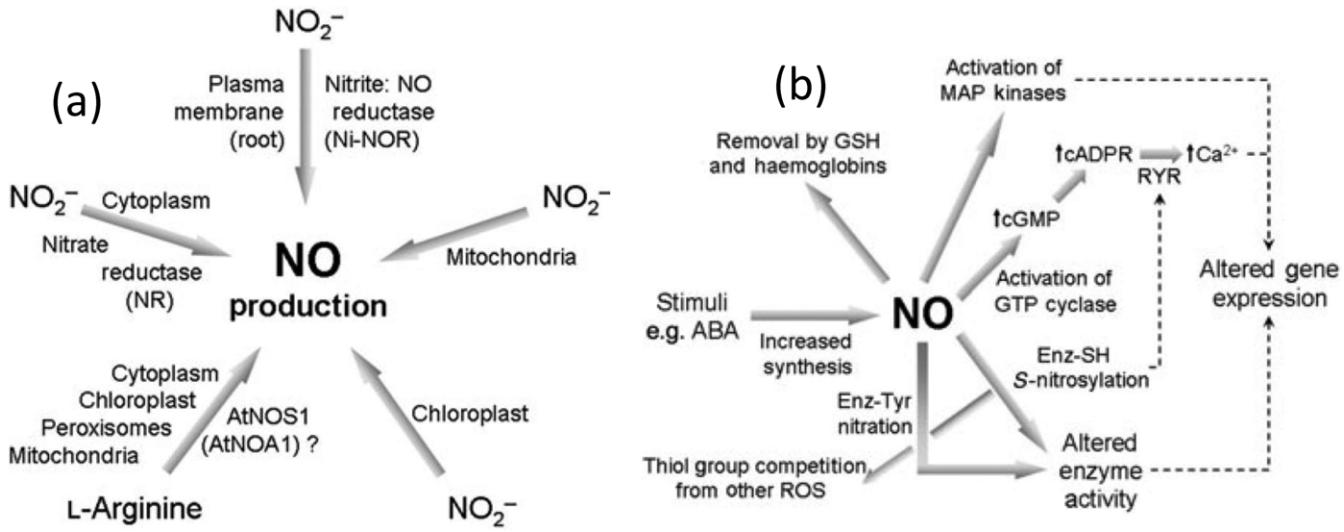

Figure 32. Following Wilson *et al* [249]. (*a*) NO can be synthesized enzymatically via NO_2^- and nitrate reductase (NR) and by L-arginine-dependent NO synthase (NOS); (*b*) Some NO signalling pathways in plants: NO induces cGMP increases that in turn increase Ca^{2+}. This and activation of mitogen-activated protein (MAP) kinase pathways can result in gene expression. NO can react with protein thiol and tyrosine groups, but these groups can react competitively with other species as well.

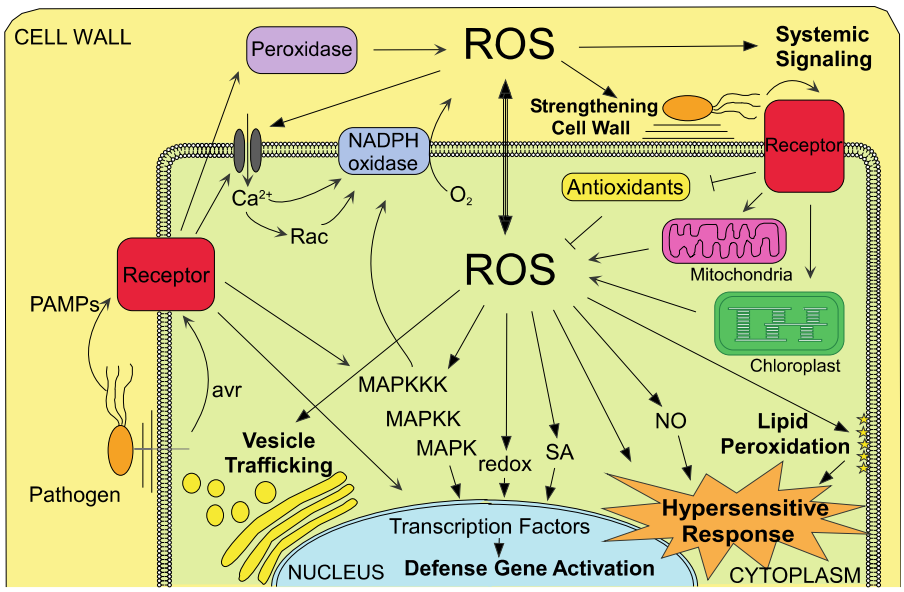

Figure 33. Following Torres *et al* [253]. When plants recognize pathogens, various signalling pathways are initiated, including production of ROS, that act in turn in many subsequent pathways.

attacks is well known to involve the creation of various RONS, in a manner analogous to animal innate immunity. The so-called plant 'oxidative burst' (in the form of O_2^-) in response to pathogen detection by the plant was apparently first identified by Doke [252] in potato tuber disks. Figure 33 illustrates (following Torres [253]) the central role played by RONS (mostly focused on ROS here) in plant defence. For example, figure 33 shows that ROS are created both inside and outside the plant cell in response to pathogen detection, and that a suite of responses are activated, including the so-called 'hypersentive response' (HR). HR, a kind of programmed cell death, involves the ROS-induced death of a small number of cells at the site of infection [254]. ROS are also known to act to reinforce cell walls, initiate various forms of systemic signalling as well as activate transcription factors to create new enzymes. Cell wall reinforcement occurs, in some cases at least, through 'lignification,' and this has been suggested to be due to H_2O_2 accumulation [255]. In part, the ROS generated are also thought to actively kill invading pathogens, but perhaps more importantly, they act as signalling molecules, described in greater detail below. It is worth noting that RONS act as a kind of chemical messenger between plants and microorganisms, including when the interactions are symbiotic [256].

As Wojtaszek [254] points out, there appear to be significant similarities between the 'respiratory burst' in animal innate immunity and the 'oxidative burst' in plants, but there are differences as well. Plant cells are less mobile (there are no macrophages, for example, in plants) and the plant cell wall is

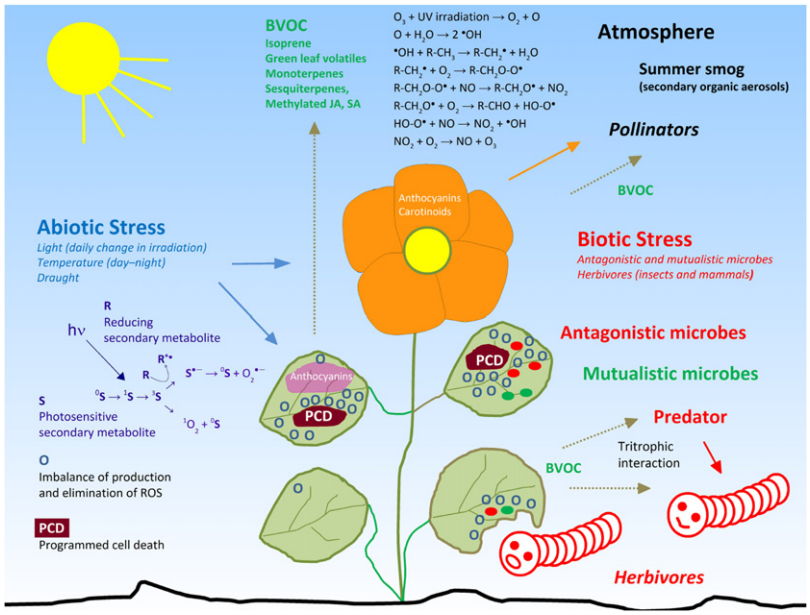

Figure 34. Following Hadacek *et al* [247]. Above ground, stresses originating from both biotic and abiotc sources cause the plant to generate ROS. These cause signal cascades to aimed at enhancing plant survival. RONS interact with plants below ground as well.

much more rigid than for animals. Generally, the phagocytic-generated RONS in animals are confined to the vacuoles within which the invading microbes are confined, and the phagocytes survive the RONS-effected phagocytotic process. In plants, the HR involves local cell death, as noted above.

6.3. RONS signalling in plants

Gechev *et al* [257] point out that the variations of RONS in time and space in plants act not only for pathogen response and cell death, but also as signals for growth, development and abiotic stress response. For example, Foreman *et al* [258] and Carol and Dolan [259] illustrate how NADPH oxidase plays a key role in stimulating plant cell expansion. These authors' discussion of how RONS play these roles mirrors in some ways the previous discussion on animal physiology. Hadacek *et al* describe the special case of ROS interacting with plants both above and below ground [247]. Figure 34 illustrates these authors' view of above-ground plant-ROS interactions, including both biotic and abiotic sources of stress. Light-generated ROS, antagonistic microbes, predators and herbivores all contribute to induce ROS plant stress. The effects of these exogenous sources of oxidative stress include impacts on plant signalling specificity and reversibility and these are related to chemical identity, dose, location, plant history and development stage, and interactions with other signalling agents, such as hormones, for example the lipid jasmonate [260]. Interactions between plant lipids and RONS are discussed below.

A recent development in the plant biology literature involves the intriguing capacity of plant RONS signalling to propagate in waves [123, 261]. Miller *et al* [261] visualized RONS signals travelling at a rate of about 8 cm min^{-1}. There is evidence that such signalling waves involving RONS also occur in animals—in mitochondria for example, as discussed by Zhou *et al* [262]. And there seems to be a connection with RONS and radiation damage: Mikkelson and Wardman [122] discussed a mitochondria-to-mitochondria transport of RONS as being responsible for this phenomenon.

6.4. Lipids and RONS in plants

One of the important connections between plant and animal physiology and the role of RONS has to do with reactive electrophile species (RES), especially lipid products. As noted earlier, RONS-lipid reactions create electrophilic species, often containing an electrophilic 'β-carbon,' as illustrated in figure 11 [74]. Malondialdehyde (MDA) is a common example of this structure and is thought to play important biochemical roles. Groeger and Freeman [74] and Farmer and Davoine [260] point out that these RES (or 'oxylipin RES') can react readily with protein thiol (or 'sulphydryls: S–H) groups, thereby altering protein structure and activity, often leading to changes in gene expression. Mueller and Berger [263] note that reactive electrophilic oxylipins can be created either enzymatically or non-enzymatically, but most pathways are non-enzymatic. Details of the roles of RES in gene expression and even for drug development are emerging [264].

6.5. Connection of plant redox biology to agriculture and food

Foyer and Noctor [14] note that... 'Cellular redox metabolism in humans and plants is intimately connected through our consumption of the plant foods that we cultivate and through human effects on the natural environment and control of agricultural conditions.' Although plant antioxidants alone are not sufficient to guarantee health as noted above, there is no controversy about the positive health effects of diets rich in vegetables and fruits. It seems that plant metabolites are somehow helping to regulate human gene transcription to

counter cancer and other inflammatory degenerative diseases. Further, because green plants use redox-sensitive light and are subject to attacks by pathogens and predators, Foyer and Noctor [14] suggest that plant redox biology is uniquely closely associated with both defence and growth responses, which in turn is reflected in the nutritional quality of their products for humans.

7. Concluding remarks

At the time of this writing (late 2011), there appears to be rapidly growing worldwide interest in the development and application of atmospheric pressure, low-temperature plasma devices for biomedical applications. The main goal of this review is to point out to plasma biomedicine researchers some of the ways by which common reactive species created in atmospheric pressure plasmas are extremely important biologically. They play key roles in such fundamental processes as cell signalling, metabolism and immunity for plants and animals. They are known to be intimately involved not only in many important human diseases and ageing, but are also known to be active in numerous established therapies. Many chemically active compounds acting either therapeutically (as 'drugs') or as toxins, as well as photons, often have their positive or negative effects mediated directly or indirectly by RONS.

The obvious challenge for this community is to learn to harness the potential therapeutic potential of plasma-generated reactive species with minimal negative side effects. But how do plasma-generated reactive species act biologically? Few details are known at present, but there are some obvious questions that demand answers. A centrally key point is that RONS generated in cells through enzymatic processes or that are generated within cells by drugs or photons will no doubt often have very different biomedical effects than RONS created in an external plasma source. Externally generated reactive species must be transported to active sites in cells and there may well be many reactions that could occur between the plasma generation and biologically active sites. How do RONS and their reaction products interact within cells and tissue in biochemical cycles, including gene expression? What reactive species at what concentration and dose should be applied at what locations and under what conditions? How can the reactive species dosage be monitored and controlled?

There are some important emerging medical challenges that plasma technology appears clearly capable of impacting, such as treatment of wounds infected with antibiotic-resistant bacteria, mimicking the innate immune response to infection. Acting as a topical, pleioitropic agent (i.e. acting via multiple pathways), plasma-generated RONS should minimize resistance development. There is a growing realization that recent efforts to develop systemic antibiotics by targeting specific enzymatic pathways have failed, at least in part, because they don't sufficiently attack broader metabolic and signalling networks [265]. As Nathan [72] points out, the properties of RONS are such that they can '... kill other cells when high levels impose maladaptive signaling at a global level. This is just one manifestation of a phenomenon that is widespread in biology: the propensity for one organism to exploit or attack another by forcing its opponent to contain or express signaling molecules at levels inappropriate for the time and place.' There are no doubt good biological reasons that RONS have been used to fight infections by immune systems of many species for many millions of years.

Although often cited as a possible or probable mechanistic cause of their biomedical efficacy, reactive oxygen and nitrogen species have generally only been invoked indirectly and generically or in an *a posteriori* fashion in explaining plasma biomedicine results. It is true that this tendency is beginning to change as plasma researchers begin to dig deeper into plasma-generated RONS-induced biochemical mechanisms (e.g. [266, 267]). But generally, little real thinking has been done yet about the important question of how RONS applied from an external (plasma) source might impact living tissue and cells in a medically positive way. The example of nitrated fatty acids and other reactive electrophilic lipids, noted several times in the paper, offer a compelling possible example. It is known that plasma-generated species (NO_x, especially in an acidic environment) will react with endogenous unsaturated fatty acids to form nitrated reactive electrophiles. It is also known that these species can induce anti-inflammatory gene expression pathways [268]. In some cases, these reactive electrophiles are known to be relatively unstable and difficult to make into a drug [269]. If it were possible to create such species locally, for example, with an endoscopic form of plasma delivery, this might represent a unique therapeutic opportunity for plasma biomedicine. The general observation is that plasma-generated species are likely to exert their effects indirectly, and it will be the more stable secondary products such as oxidized or nitrated lipids, proteins or carbohydrates that are the most probable biochemically active species.

In any case, it is fortunate for plasma biomedicine that the technology is being widely investigated at precisely the time when redox biology is one of the 'hottest' topics in aerobic biology. The plasma biomedicine research community should be able to exploit this explosion of general biomedical interest and attention to help *define* genuinely focused, hypothesis-driven studies; to suggest *diagnostic methods* to use in conducting these studies; and to provide a *powerful scientific context* within which the results can be interpreted. In addition, by orienting plasma biomedicine in this rich vein of current biology research, the nascent field will, it is hoped, gain credibility and visibility in the wider biomedical research community.

The challenges facing plasma biomedicine should not be underestimated. As noted in the introduction, plasmas are themselves far from fully understood and our limited understanding of biology certainly does not need to be explained to anyone. Plasmas often deliver their suite of chemical species to tissue with accompanying fields, currents, charges and photons. Plasma chemistries are notorious for depending very sensitively on gas precursors and many other parameters and atmospheric pressure plasmas are especially spatially inhomogenous and time-varying. Synergies are no doubt present and may be dominant. Biological samples and subsequent assays may vary from experiment to experiment due to inherent, uncontrollable fluctuations. Research

using animal or human subjects greatly increases costs and (thoroughly justifiable) regulatory barriers make progress slower than corresponding studies on non-animal targets. The relatively low cost of plasma devices and the corresponding low entry barrier may encourage untrained and/or unethical individuals to try to exploit the field for personal gain but in the process create problems for the rest of the field. By contrast, real practical progress will eventually demand large-scale clinical trials and this will depend on attracting the interest and financial support of companies whose 'interest threshold' may not be attained without the likely prospect of generating an attractive 'financial threshold.' All of these factors, and probably others as well, will tend to stretch timescales for widespread adoption of future plasma biomedical technologies. Only time and experience will show what those timescales will be, but corresponding trajectories for roughly analogous technologies like lasers in medicine and PDT were on the order of decades. The plasma medicine community should probably prepare itself for something similar.

Daunting though the aforementioned warnings and provisos may be, this review must end on a more optimistic note. It is commonly observed that progress in science and technology often occurs at boundaries between seemingly unrelated fields. Surely the overlap of low-temperature plasma-generated RONS and redox biology and medicine falls well within this category; this perhaps augurs well for the future of the field.

Acknowledgments

The author wishes to thank Mark Kushner, Lee Riley, Matt Traylor, Cameron Abrams, Mike Lieberman, Matt Pavlovich and Hiroshi Nikaido for helpful comments on the manuscript. In addition, numerous discussions involving plasma biomedicine with UC Berkeley colleagues Douglas Clark, Jeffrey Reimer, Yukinori Sakiyama, Sharmin Karmin, Ting-Ying Chung and a host of undergraduate researchers; Greg Morfill and colleagues at the Max Planck Institute for Extraterrestrial Physics in Garching, Germany; and many other plasma research colleagues around the world are very much appreciated. Much of the paper was written during a 3-month period supported by the Nanoscience Foundation in Grenoble, France, in 2011.

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