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Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms

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In the past few years, nuclear DNA damage-sensing mechanisms activated by ionizing radiation have been identified, including ATM/ATR and the DNA-dependent protein kinase. Less is known about sensing mechanisms for cytoplasmic ionization events and how these events influence nuclear processes. Several studies have demonstrated the importance of cytoplasmic signaling pathways in cytoprotection and mutagenesis. For cytoplasmic signaling, radiation-stimulated reactive oxygen species (ROS) and reactive nitrogen species (RNS) are essential activators of these pathways. This review summarizes recent studies on the chemistry of radiation-induced ROS/RNS generation and emphasizes interactions between ROS and RNS and the relative roles of cellular ROS/RNS generators as amplifiers of the initial ionization events. Cellular mechanisms for regulating ROS/RNS levels are discussed. The mechanisms by which cells sense ROS/RNS are examined in terms of how ROS/RNS modify protein structure and function, for example, interactions with metal–thiol clusters, protein tyrosine nitration, protein cysteine oxidation, S-thiolation and S-nitrosylation. We propose that radiation-induced ROS are the initiators and that nitric oxide ($NO^{\bullet}$) or derivatives are the effectors activating these signal transduction pathways. In responding to cellular ionization events, the cell converts an oxidative signal to a nitrosative one because ROS are too reactive and unspecific in their reactions for regulatory purposes and the cell is equipped to precisely modulate $NO^{\bullet}$ levels.
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Introduction

Exposure of cells to clinically relevant doses of ionizing radiation causes significant nuclear DNA damage: 1000 single-strand breaks, 40 double-strand breaks and 3000 damaged bases per gray (Gy) (Ward, 1994). Sensing mechanisms within the nucleus detect this damage and initiate signal transduction pathways, resulting in activation of cell cycle checkpoints and DNA damage repair. In the past few years, components of these nuclear DNA damage-sensing mechanisms, including ATM/ATR, DNA-dependent protein kinase, and CHK1/2 kinases and their homologs from yeast to man, have been identified (Durocher and Jackson, 2001; Khanna *et al*., 2001; Shiloh and Kastan, 2001).

Less is known about the consequences of cytoplasmic irradiation and how cytoplasmic ionization events influence nuclear processes. Nonetheless, there is a significant body of published information indicating the importance of radiation-stimulated cytoplasmic signaling in terms of cytoprotection and mutagenesis. Ionizing radiation activates cytoplasmic signal transduction pathways involved in cell proliferation and anti-apoptotic mechanisms, including growth factor receptors, changes in cytoplasmic Ca^{2+} levels and stress-response kinases (e.g. Hallahan *et al*., 1991; Haimovitz-Friedman *et al*., 1994; Stephenson *et al*., 1994; Todd and Mikkelsen, 1994; Kasid *et al*., 1996; Kavanagh *et al*., 1998; Tuttle *et al*., 1998; Schmidt-Ullrich *et al*., 2000). Alpha-particle microbeam irradiation of cytoplasm elicits a spectrum of mutations different from that obtained with nuclear radiation and reflective of the type of mutations spontaneously produced by endogenous metabolism (Wu *et al*., 1999).

An important question that arises is how the few primary ionization events produced at clinically relevant doses (approximately 2000/Gy/cell) are amplified to account for the rapid and robust activation of cellular signal transduction pathways (Ward, 1994). Even considering secondary free radical products resulting from the initial ionization, the calculated amount of reactive oxygen species (ROS) generated is relatively insignificant compared to the amount produced by metabolism (Ward, 1994; see below). However, recent studies indicate that cells are endowed with cytoplasmic amplification mechanisms involving ROS/RNS (reactive nitrogen species) and responsive to low doses of ionizing radiation (e.g. Clutton *et al*., 1996; Narayanan *et al*., 1997; Morales *et al*., 1998; Leach *et al*., 2001, 2002). These mechanisms appear to be part of general cellular response pathways to oxidative stress (e.g. Ichas and Mazat, 1998; Marshall *et al*., 2000; Zorov *et al*., 2000; Droge, 2002; Paxinou *et al*., 2001). Our intent in this review is to discuss the overall chemistry of free radical

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production in terms of what ROS/RNS are produced by cellular metabolism and following a radiation exposure, describe possible cellular radiation-induced ROS-sensing mechanisms, and present possible mechanisms emphasizing regulatory protein modification by which an initial radiation-induced ionization event is transduced into a cellular response. Reaction rates, relative intracellular concentrations of ROS/RNS and their subcellular localization are assessed in evaluating biological relevance. What emerges from this analysis is the concept that although ROS are the initial reactants produced from an ionization event, RNS are the actual effectors/activators of redox-dependent cellular signal transduction pathways.

Oxidation and nitration initiated by superoxide ($^{\bullet}O_2^-$) and nitric oxide ($NO^{\bullet}$)

Although the overall chemical biology of oxidative and nitrosative stress remains complex and ill defined, many individual chemical reactions are quite well characterized. We review here the main reactions, focusing on protein oxidation/nitration and the potential basis for signaling pathways involving short-lived free radicals.

'Oxidative stress' is a term associated with both enhanced production of ROS and reduced efficacy of protection by antioxidant enzymes or low molecular weight antioxidants. After the discovery of $NO^{\bullet}$ as a biological entity (Arnold *et al*., 1977; Palmer *et al*., 1987), and especially the realization (Beckman *et al*., 1990) that superoxide radicals ($^{\bullet}O_2^-$) and $NO^{\bullet}$ form more powerful or 'cloaked' oxidants (Koppenol *et al*., 1992) via $ONOO^-$ formation, oxidative stress has become unavoidably linked to 'nitrosative stress' and RNS. A further complication is the involvement of carbon dioxide as a major potential modifier of nitrosative stress, via its interaction with $ONOO^-$ to produce carbonate radicals, which had previously not been considered in a biological context (Lymar and Hurst, 1995). The key species associated with oxidative and nitrosative stress and the main routes of interaction between the species are shown in Figure 1.

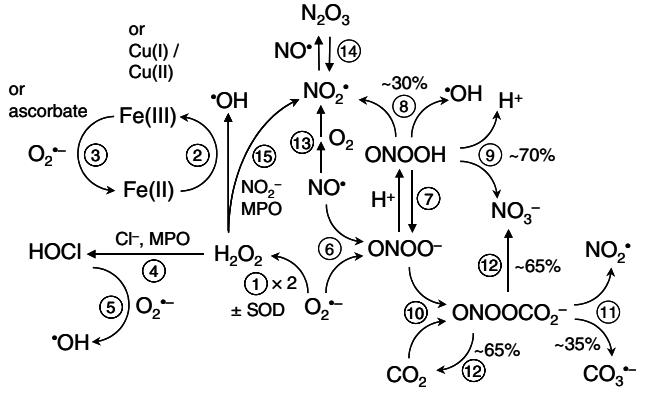

Figure 1 Main pathways for the formation of reactive oxidants from $^{\bullet}O_2^-$ and $NO^{\bullet}$ radicals

Prior to 1990, oxidative stress was exclusively focused on the production of $^{\bullet}O_2^-$ and more reactive oxidants derived from $^{\bullet}O_2^-$ involving redox catalysts (Halliwell and Gutteridge, 1999). While $^{\bullet}O_2^-$ radicals disproportionate quite rapidly to form hydrogen peroxide in a pH-dependent reaction (Bielski *et al*., 1985), superoxide dismutase (SOD) efficiently catalyzes the reaction (Fridovich, 1995) (Figure 1, reaction 1) and thus is a major controller of the steady-state concentration of $^{\bullet}O_2^-$. Hydrogen peroxide presents a potential oxidative challenge, although it is efficiently deactivated by glutathione peroxidase and catalase. However, in the presence of trace redox metals (principally iron and copper), production of highly reactive hydroxyl radicals via Fenton chemistry (Figure 1, reaction 2) can enhance damage. Note that only catalytic levels of redox metal are required for this pathway, since $^{\bullet}O_2^-$ itself, or alternative reductants such as ascorbate, 'recycle' the oxidized metal to the active, reduced form (reaction 3). A recent study demonstrated colocalization of a local Fenton reaction (or at least, oxidation of dihydrorhodamine; see below) and iron-containing mass-dense particles in hepatocytes (Kietzmann *et al*., 2000).

Another catalyzed transformation of hydrogen peroxide to a more reactive oxidant involves chloride and myeloperoxidase to form hypochlorous acid/hypochlorite ($HOCl/ClO^-$, reaction 4). The latter species are both oxidants and chlorinating agents (Winterbourn and Kettle, 2000); HOCl (but not ClO^-; Long and Bielski, 1980), pK_a HOCl = 7.5), reacts rapidly with $^{\bullet}O_2^-$ to form hydroxyl radicals (Candeias *et al*., 1993) (reaction 5). Owing to the high reactivity of HOCl, its steady-state concentration is likely to be generally low. Therefore, except in activated phagocytes (where high concentrations of $^{\bullet}O_2^-$ are also formed), reaction 5 seems unlikely to be a significant source of $^{\bullet}OH$ radicals.

Hydroxyl radicals are highly reactive (Ross *et al*., 1998) – 'promiscuous' is a term often seen – but high reactivity implies low selectivity and diffusion distance, and inability to act as a cellular messenger. $NO^{\bullet}$ is chemically rather unreactive toward most cellular constituents except for the heme centers that are its prime biological targets in controlling vascular tone (Ignarro, 2000), but $NO^{\bullet}$ reacts with $^{\bullet}O_2^-$ on every collision (Nauser and Koppenol, 2002) to form $ONOO^-$ (Figure 1, reaction 6). This 'diffusion-controlled' reaction will usually not outcompete the somewhat slower SOD-catalyzed dismutation, reaction 1. This is because the relative importance of the two reactions as 'sinks' for $^{\bullet}O_2^-$ depends also on the steady-state concentrations of $NO^{\bullet}$ and SOD. Leaving aside cellular heterogeneity, SOD levels are usually several micromolar (Fridovich, 1995; Halliwell and Gutteridge, 1999) whereas steady-state $NO^{\bullet}$ levels are probably considerably lower except under some pathological conditions (Ignarro, 2000). The concentrations of $NO^{\bullet}$ needed to saturate heme targets are nearer nanomolar (Bellamy *et al*., 2002). However, particularly under conditions where $NO^{\bullet}$ synthases (NOS) are stimulated, $ONOO^-$ formation (reaction 6) may become important. This may explain the enhanced

tyrosine nitration of the mitochondrial enzyme, Mn-SOD, following radiation exposure of tissue culture cells (Leach *et al*., 2002). Other mechanisms involving myeloperoxidase and discussed below may need to be invoked to explain the enhanced tyrosine nitration of Mn-SOD observed *in vivo* in rejected kidney allografts (MacMillan-Crow *et al*., 1996).

Peroxynitrite exists in fast, dynamic equilibrium with its conjugate acid (ONOOH) under physiological conditions (pK_a ONOOH = 6.8, Figure 1, equilibrium 7) (Radi *et al*., 2000). In the absence of carbon dioxide, $ONOO^-$/ONOOH decays via the protonated form, reactions 8 and 9 ($ONOO^-$ is stable at high pH). The minor pathway 8 produces reactive $NO_2^\bullet$ and $^\bullet OH$ radicals, while a majority of ONOOH decomposes harmlessly to nitrate, reaction 9. Nitrogen dioxide ($NO_2^\bullet$) is *much* more reactive than $NO^\bullet$ toward common cellular constituents such as thiols (Wardman, 1998; Kirsch *et al*., 2002). The effective half-life of $ONOO^-$/ONOOH at pH 7.4, 37 °C is ~0.7 s in the absence of other reactants (Radi *et al*., 2000). This half-life is important because it enables us to assess the importance of alternative pathways for $ONOO^-$ decomposition. In particular, carbon dioxide reacts with $ONOO^-$ sufficiently fast that under physiological levels of carbon dioxide, reaction 10 to form an unstable intermediate ($ONOOCO_2^-$), has a half-life of only ~0.1 s (Lymar and Hurst, 1995), and is thus the major route for $ONOO^-$ disappearance *in vivo*. Whether experimental models accurately mimic $ONOO^-$ decay pathways in biology thus depends, for example, on whether bicarbonate/carbon dioxide buffer systems are used.

The importance of carbon dioxide as a new (or at least, unexpected) reaction partner in free radical biology is that the carbon dioxide/$ONOO^-$ adduct formed in reaction 10 is very short-lived (< 3 ms; Lymar *et al*., 1996), and a substantial fraction decomposes (reaction 11) to yield $NO_2^\bullet$ and the carbonate radical, $^\bullet CO_3^-$, recently described (Augusto *et al*., 2002) as 'two emerging radicals in biology'. About two-thirds of the adduct $ONOOCO_2^-$ decomposes to NO_3^- (nitrate) and carbon dioxide (reaction 12). Carbonate radical was previously only considered as a biologically important species via reaction of $^\bullet OH$ with bicarbonate/carbonate in competition with many other, more reactive cellular constituents; however, the reactivity of $^\bullet CO_3^-$ has been quite well established using radiation and photochemical methods (Ross *et al*., 1998). It is often a rather more reactive one-electron oxidant than $NO_2^\bullet$ (Ross *et al*., 1998; Augusto *et al*., 2002).

$NO_2^\bullet$ can be produced via $NO^\bullet$ not involving the $^\bullet O_2^-$ pathway to $ONOO^-$, reaction 6. In aerobic systems, reaction between $NO^\bullet$ and oxygen very probably involves $NO_2^\bullet$ as an intermediate, but the reaction is complex (see Wardman, 1998), and the rate is proportional to the *square* of the $NO^\bullet$ concentration as well as the oxygen concentration. Thus, formation of $NO_2^\bullet$ via reaction 13 is far less likely when the steady-state concentrations of $NO^\bullet$ are sub-micromolar and at physiological oxygen tensions than in many laboratory experiments where the steady-state $NO^\bullet$ concentrations might rise to tens of micromolar and the oxygen tension is around an order of magnitude higher than in tissues. The corollary is that numerous laboratory studies that have utilized convenient chemical sources of $NO^\bullet$, such as the more rapidly decomposing 'NONOates' (Fitzhugh and Keefer, 2000), in solutions equilibrated with atmospheric air (Schmidt *et al*., 1997) may well be misinterpreted because of the generation of significant quantities of $NO_2^\bullet$ and the consequent depletion of cellular thiols and other antioxidants, as well as much more protein nitration than might arise via the $^\bullet O_2^-$/$NO^\bullet$ pathway (Wardman, 1998). A further consequence of working with nonphysiological concentrations of both $NO^\bullet$ and oxygen is that the resulting nonphysiological $NO_2^\bullet$ concentrations can lead to N_2O_3 formation by equilibrium 14. The latter is a potent nitrosating agent (Williams, 1988), but whether it is formed to a significant extent in biology, except perhaps in hydrophobic regions of protein- or lipid-rich environments (e.g. membranes), has been questioned (Ford *et al*., 2002a). Both $NO^\bullet$ and $NO_2^\bullet$ partition from aqueous to lipid-rich compartments, so reactions 13 and 14 are favored in lipophilic regions (Liu *et al*., 1998), and N_2O_3 formation is plausible at the membrane/cytosol interface (Ramachandran *et al*., 2001). Much the same considerations apply to extrapolating results from experiments involving the bolus addition of $ONOO^-$. Williams (1997) concluded that 'there is no experimental evidence… that ONOOH or $ONOO^-$ can act as direct electrophilic nitrosating agents', pointing to N_2O_3 (via $NO^\bullet + NO_2^\bullet$, as above) as 'a more likely possibility'. However, direct reaction of $ONOO^-$ with some cellular targets is possible (Arteel *et al*., 1999; Crow, 2000; see below).

$NO_2^\bullet$ can be formed in a pathway involving neither $ONOO^-$ as an intermediate nor atmospheric oxidation. Nitrite (NO_2^-) is a substrate for peroxidases, especially myeloperoxidase (Burner *et al*., 2000), and protein nitration via $NO_2^\bullet$ generated from the peroxidase-catalyzed oxidation of NO_2^- by H_2O_2 as cofactor is another important pathway (Eiserich *et al*., 1988; Baldus *et al*., 2002) (Figure 1, reaction 15). Spatial mapping showed that myeloperoxidase immunoreactivity strongly colocalized with nitrotyrosine formation (Baldus *et al*., 2002). Peroxynitrite and myeloperoxidase 'leave the same footprint in protein nitration' (Kettle *et al*., 1997). Kinetic arguments show that this is much more likely to involve $NO_2^\bullet$ generated from NO_2^- by myeloperoxidase than reaction with HOCl (Kettle *et al*., 1997).

Figure 1 is not exhaustive in its mapping of reaction pathways: radical–radical reactions are often facile, and several of relevance have been characterized (Ross *et al*., 1998). Thus, $NO_2^\bullet$, as well as $NO^\bullet$, is reactive toward $^\bullet O_2^-$, producing peroxynitrate ($ONOO^-$) rather than peroxynitrite ($ONOO^-$). However, radical–radical reactions involving $NO_2^\bullet$ and $^\bullet OH$, for example, are unlikely to be of biological importance because of the extremely low steady-state concentrations of such reactive, and therefore short-lived, radicals. Figure 1 also does not show the routes of formation of $^\bullet O_2^-$ and $NO^\bullet$, which

are outlined briefly below in considering cellular homeostasis of these species.

Protein nitration: 'How the radicals do the job' (Goldstein *et al.*, 2000)

Tyrosine is a common target of nitrosative stress: 3-nitrotyrosine residues have been detected in numerous human diseases and animal models of disease and after cell irradiation (Ischiropoulos, 1998; Leach *et al.*, 2002). Figure 2 illustrates the key features of how 3-nitrotyrosine can be formed via radical pathways (Lymar *et al.*, 1996; Goldstein *et al.*, 2000; Hodges *et al.*, 2000; Lymar, 2001; Radi *et al.*, 2001). As with the use of fluorescent probes for ROS/RNS (see below), it is often overlooked that even though a target or probe eventually produces a stable ('spin paired') molecule from a radical, the reaction must involve a radical intermediate. It is equally important to recognize that the first step – the formation of a phenoxyl or tyrosyl radical – can be accomplished by diverse one-electron oxidants. These may be simple free radicals, as shown, but radicals can be formed via other routes during enzyme catalysis. Tyrosyl radicals are long lived in proteins because of reduced capacity for radical–radical reactions; the radical in ribonucleotide reductase is stable for days (Hoganson *et al.*, 1996). The enzyme prostaglandin H synthase-2 forms a tyrosyl radical during catalysis of prostaglandin formation (Gunther *et al.*, 2002). Reaction of this tyrosyl radical with $NO^{\bullet}$ leads to tyrosine nitration but does not involve formation of the tyrosyl radical by $^{\bullet}OH$, $NO_2^{\bullet}$ or $^{\bullet}CO_3^{-}$.

In Figure 2, we see that 3-nitrotyrosine can be generated from the phenoxyl radical by either $NO_2^{\bullet}$ or $NO^{\bullet}$. The latter pathway involves the intermediate formation of nitrosotyrosine, which is oxidized (e.g. by peroxidase compound I or II intermediates) to an iminoxyl radical and thence to nitrotyrosine (Gunther *et al.*, 2002). Rate constants for reaction of both $NO^{\bullet}$ and $NO_2^{\bullet}$ with the tyrosine phenoxyl radical are equally high (Prütz *et al.*, 1985; Eiserich *et al.*, 1995). Generating

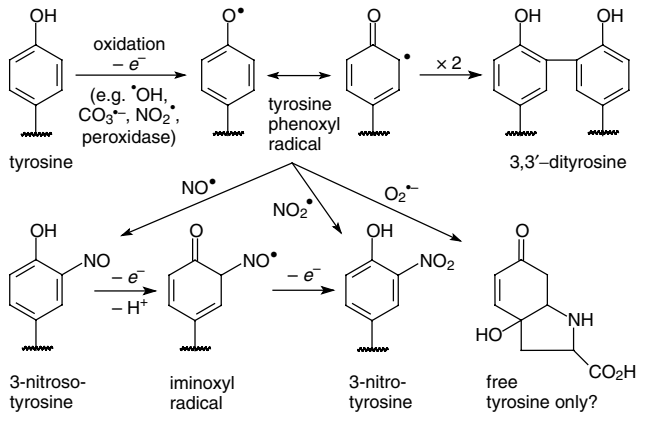

Figure 2 Reaction pathways in tyrosine oxidation/nitration

$NO^{\bullet}$ and $^{\bullet}O_2^{-}$ simultaneously in varying ratios or adding $ONOO^{-}$ to tyrosine yields nitrotyrosine and dityrosine but "all the experimental results can be explained in terms of free radical chemistry" (Hodges *et al.*, 2000), that is, without direct reaction of tyrosine with $ONOO^{-}$ or its CO_2 adduct.

A key factor in the enhancement of tyrosine nitration by CO_2 is the ~100-fold higher reactivity of the carbonate ($^{\bullet}CO_3^{-}$) radical (compared with $NO_2^{\bullet}$) toward tyrosine at physiological pH (Ross *et al.*, 1998). Thus, the first step in the two-stage nitration process is accomplished faster in the presence of CO_2 via $^{\bullet}CO_3^{-}$ production from $^{\bullet}O_2^{-}/NO^{\bullet}/CO_2$ (Figure 1, reactions 6, 10, 11). Too high a flux of $^{\bullet}O_2^{-}$ can diminish tyrosine nitration in model experiments, because the phenoxyl radical reacts rapidly with $^{\bullet}O_2^{-}$ (Jin *et al.*, 1993). The rate constant for reaction of $^{\bullet}O_2^{-}$ with $NO_2^{\bullet}$ to form peroxynitrate ($ONOOO^{-}$) is also high (Ross *et al.*, 1998), but the steady-state concentration of the latter is too low in biology for this radical–radical reaction to be important. It may be a feature in model experiments with oxidation of leuco dyes (Jourd'heuil *et al.*, 2001) or vicinal diamine probes for $NO^{\bullet}$ (Espey *et al.*, 2002) (see below) when similar considerations apply.

In Figure 2, two decay pathways for tyrosyl radicals not involving $NO^{\bullet}$ or $NO_2^{\bullet}$ are also shown. These can be viewed as arising from the resonance form of the radical shown to the right of the phenoxyl radical. It might be expected that radical–radical coupling to form fluorescent dityrosine, while facile with free tyrosine (Prütz *et al.*, 1983), is less likely to occur in proteins. However, protein–protein crosslinks have been observed via radical-induced tyrosine oxidation (Hashimoto *et al.*, 1982). Whether the product shown in Figure 2, arising from the fast reaction between tyrosyl and $^{\bullet}O_2^{-}$ radicals (Jin *et al.*, 1993), is formed with tyrosine residues in proteins is not known.

Problems in the use of fluorescent probes for reactive oxygen/nitrogen species

Probably, thousands of studies have used molecular probes for reactive intermediates in oxidative and nitrosative stress, probes that typically show weak fluorescence in their reduced form but fluoresce strongly on oxidation. While of undoubted value, the interpretation of such experiments must be approached with caution. Figure 3 presents simplified schemes for formation of the oxidized forms of two common types of probe, as well as the basis for several probes reactive toward $NO^{\bullet}$ (or at least, reactive once activated). Reduced (dihydro) fluoresceins, or fluorescins, are the most common probes, especially the dichlorinated form, $DCFH_2$ (Ischiropoulos *et al.*, 1999). As are most phenols, they are rapidly oxidized by $^{\bullet}OH$, $NO_2^{\bullet}$ and $^{\bullet}CO_3^{-}$ radicals (Wardman *et al.*, 2002), forming phenoxyl radicals that may disproportionate to form an oxidized quinoid structure and parent dye. The

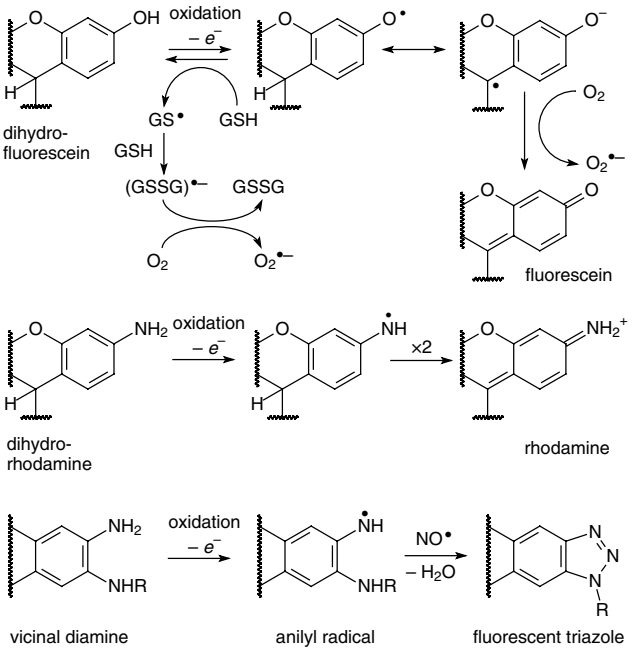

Figure 3 Chemical pathways in the detection of reactive oxygen and nitrogen species using common fluorescent probes. Dihydrofluoresceins and dihydrorhodamines are general probes for oxidizing species; the intermediate free radicals are thiol reactive and can initiate a chain reaction generating further $^{\bullet}O_2^-$ as shown. Vicinal diamines are probes for nitric oxide, but require prior oxidation to a reactive intermediate

extensive resonance in the quinoid structure (not shown) results in strong fluorescence. Obviously, $DCFH_2$ or similar molecules are good probes for *nonspecific* free radical oxidants.

However, there are major problems with their use. The first is that the oxidation is very slow with H_2O_2, but fast in the presence of peroxidase and similar catalysts (Ischiropoulos *et al*., 1999). In this class, the most important is cytochrome *c*, which catalyzes the oxidation of $DCFH_2$ at concentrations as low as a few nanomolar in a model system (Burkitt and Wardman, 2001). At a constant flux of $^{\bullet}O_2^-$ radicals from xanthine/xanthine oxidase, the fluorescence signal was proportional to the cytochrome *c* concentration in the 1–25 nM range; with 10 nM cytochrome *c*, fluorescence was almost invariant over an $\sim$20-fold range of $^{\bullet}O_2^-$ production (Burkitt and Wardman, 2001). The importance of these observations is that cytochrome *c* is released from mitochondria to cytosol during apoptosis (Yang *et al*., 1997). Hence, apparent modulation of oxidative stress suggested by changes in fluorescence of $DCFH_2$ might reflect apoptotic pathways rather than real changes in ROS or RNS.

Another problem is the generation of $^{\bullet}O_2^-$ radicals, either by the radical intermediate reducing oxygen (Rota *et al*., 1999a), or the peroxidase-catalysed formation of a phenoxyl radical center on the remaining phenol group in the oxidized dye (not shown in Figure 3), reacting with glutathione (GSH) to form thiyl radicals (Rota *et al*., 1999b). The latter can reduce oxygen via the well-known 'redox switch' involving thiol conjugation as shown (Wardman, 1999). It is important to note that the reactions involving radicals with either oxygen or thiols need not be thermodynamically favored to proceed: removal of products from an unfavorable equilibrium can rapidly drive an overall reaction (Wilson *et al*., 1986). The oxidized, fluorescent dye can also be photo-reduced, a potential problem in measurement (Marchesi *et al*., 1999). While it might be regarded that the 'self-amplification' of signal resulting from dye radical chemistry is a bonus, quantitative interpretation is fraught with difficulty.

The same factors are probably a feature of the chemistry of the rhodamine dyes that are less well characterized (Figure 2). Whether the radicals disproportionate or react with oxygen to form the fluorescent, oxidized form will depend on kinetic and redox properties. Other difficulties that arise with these dyes include photo-oxidation. For dyes that are esterified and become sequestered upon action of cytoplasmic esterases, dye leakage from the cell and normalization of intracellular dye concentrations represent additional problems (e.g. Leach *et al*., 2002).

Peroxidase chemistry is also a factor in the use of vicinal diamines to identify $NO^{\bullet}$ production in biology. 1,2,-Phenylenediamine, 2,3-diaminonaphthalene and the newer diaminofluorescein dyes (e.g. DAF-2; Kojima *et al*., 1998) or their rhodamine analoges (Kojima *et al*., 2001) all require prior activation by nonspecific oxidants before reaction with $NO^{\bullet}$. Thus, all these share a common reaction scheme in which a fluorescent triazole is eventually formed from $NO^{\bullet}$, but requires an obligate anilyl radical intermediate (Figure 3) (Uppu and Pryor, 1999). This intermediate can obviously be formed from a wide variety of radical and enzyme-based oxidants, the levels of which will modulate the signal (Jourd'heuil, 2002). With $NO^{\bullet}$ in aerobic environments, aerobic oxidation of $NO^{\bullet}$ to $NO_2^{\bullet}$ will provide such an oxidant. However, relying on this for quantitative measurements in biological systems introduces uncertainties because the levels of competing reactants for $NO_2^{\bullet}$, particularly thiols, urate and ascorbate, might not be constant (Ford *et al*., 2002a).

It is noteworthy that other probes suffer from different problems: the EPR-detectable probe carboxy-'PTIO' (an imidazoline-3-oxide-1-oxyl derivative) reacts with $NO^{\bullet}$, but it is thought that $NO_2^{\bullet}$ is produced (Hogg *et al*., 1995; Pfeiffer *et al*., 1997), which oxidizes cellular thiols in microseconds (Ford *et al*., 2002a). This probe was used in a study of an $NO^{\bullet}$-mediated bystander response (Matsumoto *et al*., 2001).

In summary, the different chemical probes used to detect ROS/RNS each have individual problems that make interpretation of results obtained with their use potentially ambiguous. Until better, more specific probes are developed, other correlative approaches must be applied to more definitively identify specific ROS/RNS. For example, measurements of protein nitrotyrosine and NOS activities can be used to distinguish between RNS from ROS formation.

Lifetimes of radicals and diffusion distances

An estimate for the typical diffusion distance of a species can be made if the lifetime of the species can be calculated. The root-mean-square diffusion distance x is related to the lifetime t and the diffusion coefficient D by the Einstein equation in three-dimensional space: $x = (6Dt)^{1/2}$ (the average path length can be alternatively calculated as $(16Dt/\pi)^{1/2}$ (Roots and Okada, 1975), numerically similar). Diffusion coefficients are dependent on viscosity and will generally be smaller in a biological matrix than in water, but for a small radical a value of D around $1 \times 10^{-9}\,m^2\,s^{-1}$ is reasonable (Ford *et al*., 2002a). With this value, lifetimes of $\sim 1\,\mu s$, 1 ms and 1 s correspond to diffusion distances of ~ 0.1, 2 and 80 μm, respectively.

In calculating lifetimes of species, *all* the reactions that it undergoes must be included. In most biological reactions, the concentrations of biological reactants are usually in large excess over that of the reacting radical, and the kinetics are closely exponential or first order in the concentration of the reagent in excess. The half-life then equals the natural log 2 divided by the sum of the products of rate constant and concentrations for *all* species the radical is reacting with. In this way, it was suggested that the lifetime of an $^{\bullet}OH$ radical in a cell was a few nanoseconds and its diffusion distance therefore a few nanometers (Roots and Okada, 1975).

Cell signaling by reactive oxygen/nitrogen species: a two-step hypothesis

The parallels drawn in the two-step nitration mechanism for nitrotyrosine formation, and the description above of problems with interpretation of experiments involving chemical probes for ROS/RNS, are intentional and instructive. They offer the foundation for a general, mechanism-led hypothesis of cell signaling involving radicals. In both scenarios, the first step was a nonspecific oxidative reaction, often involving highly reactive radicals with short diffusion distances, to produce a radical that in proteins is quite long-lived. (The diffusion distance of $NO_2^{\bullet}$ in the cytosol was recently estimated to be $\sim 0.2\,\mu$m (Ford *et al*., 2002a), and similar calculations for $^{\bullet}CO_3^-$ indicate about half this value because of its $\sim$ four-fold higher reactivity toward thiols compared with $NO_2^{\bullet}$.) The second step involves reaction either with radicals that are not very reactive (e.g. $^{\bullet}O_2^-$ or $NO^{\bullet}$) or with reactive radicals that are derived from species that act as carriers and so confer a much larger diffusion distance. Thus, *S*-nitrosothiols are storage forms of $NO^{\bullet}$, transporting $NO^{\bullet}$ by trans-nitrosation via thiols (Hogg, 2000). Transfer of $NO^{\bullet}$ from extracellular *S*-nitrosothiols to the cytosol may involve basically similar chemistry (Ramachandran *et al*., 2001), with N_2O_3 as a nitrosating agent at the membrane/cytosol interface. *S*-nitrosothiols have low reactivity toward $^{\bullet}O_2^-$ (Ford *et al*., 2002b), but can release $NO^{\bullet}$ in tens of seconds in the presence of micromolar levels of Cu(II) under conditions where oxidized GSH (GSSG) does not stabilize the redox metal (Noble and Williams, 2000). It is important to note that there is less than one free copper ion per cell (Rae *et al*., 1999). Interaction between *S*-nitrosothiols and thiols has been claimed to generate nitroxyl (HNO/NO^-) (Wong *et al*., 1998), since N_2O is formed. The latter product is not an unequivocal indicator for nitroxyl, since it might also be produced by metal-catalyzed formation of $NO^{\bullet}$ from nitrosothiols and by the reaction of $NO^{\bullet}$ with thiols releasing N_2O via hyponitrous acid (Hogg *et al*., 1996).

The formation of *S*-nitrosothiols such as *S*-nitrosoglutathione (GSNO) may well be linked into radical damage from ROS and $NO^{\bullet}$, because a credible route to GSNO formation is the (probably) diffusion-controlled reaction of thiyl radicals ($GS^{\bullet}$) with $NO^{\bullet}$. Nonspecific 'repair' of radical damage from ROS is likely to largely involve thiols and the production of thiyl radicals; it is easily calculated from the known equilibrium constants of conjugation of $GS^{\bullet}$ with thiolate (GS^-) and oxygen such that an appreciable fraction of $GS^{\bullet}$ remains unconjugated at equilibrium at physiological oxygen tensions, thiol concentrations and pH (Wardman, 1995; 1999; Wardman and von Sonntag, 1995).

Figure 4 illustrates two aspects of this scenario. Firstly, nonspecific radical damage, for example, from radicals such as $^{\bullet}OH$ with extremely short diffusion distances, can in effect be 'transported' to other sites via regeneration of $^{\bullet}O_2^-$, involving the thiyl/disulfide radical ion 'redox switch'. (Some carbon-centered radicals, such as those from carbohydrates, can generate $^{\bullet}O_2^-$ more directly by addition of oxygen and elimination of $^{\bullet}O_2^-$ (von Sonntag and Schuchmann, 1997)). Superoxide is rather unreactive and its diffusion distance is controlled largely by SOD and steady-state $NO^{\bullet}$ levels. While the regeneration of $^{\bullet}O_2^-$ at a site distant from the original radical damage is not strictly an amplification reaction,

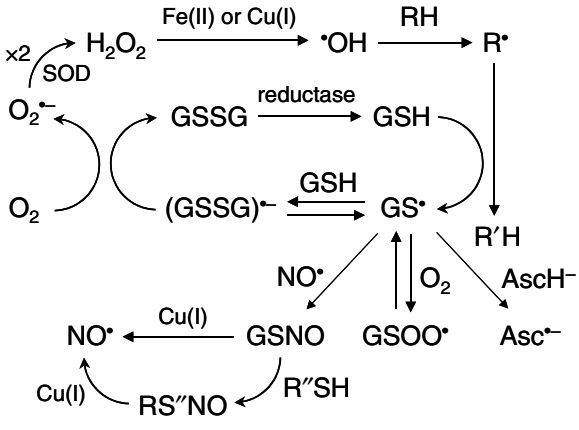

Figure 4 Outline of pathways involved in 'transporting' radical damage via regeneration of $^{\bullet}O_2^-$ or formation of GSNO, both involving thiyl radicals. 'Repair' of radical damage in the biomolecule RH may not be restituted (i.e. forming R′H) if stereochemical identity is lost in the radical. Radical 'sinks' such as ascorbate ($AscH^-$) or urate may modify pathways

it effectively increases the diffusion distance or reaction radius and facilitates possible reaction with a greater range of targets. However, it is difficult to quantitate the increased diffusion distances that might be associated with this mechanism. The lifetime of the disulfide radical-anion at physiological oxygen levels is about 30–40 μs, corresponding to a diffusion distance of around 0.5 μm. The diffusion distance of $^{\bullet}O_2^-$ will be of the same order in the presence of several micromolar SOD, but that of H_2O_2 could be much greater, since estimates of the turnover time of H_2O_2 in cells range from about 3 to 200 ms (Antunes and Cadenas, 2000). These authors have also noted that the steady-state concentrations of H_2O_2 and $^{\bullet}O_2^-$ are estimated to be in the ratio $\sim$ 100 : 1 in the mitochondrial matrix but around 1000 : 1 in the cytosol.

A second mechanism for radical-driven damage 'migration' is observed under pathological conditions or where local NO$^{\bullet}$ concentrations can exceed micromolar levels. A fraction of thiyl radicals can conceivably be trapped by NO$^{\bullet}$ and thus generate the much longer-lived 'transporter' GSNO, provided that the rate constant for reaction between GS$^{\bullet}$ and NO$^{\bullet}$ is about 10-fold higher than reaction between GS$^{\bullet}$ and GS$^-$. The extent of GSNO formation from 'repair' of nonspecific radical damage by GSH in the presence of NO$^{\bullet}$ will, however, be very dependent on other scavengers for thiyl radicals, particularly urate and ascorbate (Wardman, 1999); the latter is generally absent in cultured cells but not, of course, *in vivo*. An illustration of the expected modifying effects of ascorbate is provided in the case of interaction of the tyrosyl radical with GSH (Sturgeon *et al*., 1998).

A third mechanism for radiation-induced, radical-driven signal propagation, permissive for longer distances including *inter*cellular signaling, is described in more detail below. This too entails a two-step process with an initial nonspecific oxidative event triggering the second but specific redox sensitive step involving propagation of a reversible mitochondrial permeability transition from one mitochondrion to adjacent mitochondria (e.g. Romashko *et al*., 1998; Zorov *et al*., 2000; Leach *et al*., 2001, 2002). This propagation ultimately results in the Ca^{2+}-dependent activation of constitutive NOS and NO$^{\bullet}$ signaling (Leach *et al*., 2002).

While much work is needed to establish these concepts on a firmer quantitative basis, the main chemical pathways by which radical damage can be relocated are evident. However, signaling mechanisms are equally likely to involve control via damage amplification or by modulating a specific site. These possibilities are considered below.

Possible mechanisms for damage amplification or control involving radicals

The molecular mechanisms for true damage *amplification* are less well founded than for damage migration, except for the well-known lipid peroxidation chain reaction (Halliwell and Gutteridge, 1999) and the analogous reactions with protein (e.g. histone) hydroperoxides (Luxford *et al*., 2000), and short chain reactions observed in thiol oxidation (Lal *et al*., 1997) or on eliminating $^{\bullet}O_2^-$ from peroxyl radicals formed from oxygen adding to some carbon-centered radicals (von Sonntag and Schuchmann, 1997). The total damage initially produced in a cell by radiation, calculated on the basis of the ionization yields and by considering track recombination and high scavenging capacity, is around 1 μM/Gy. For comparison, mitochondrial respiration alone generates hundreds of millimoles of $^{\bullet}O_2^-$ per day in a human, that is, of the order of 50 nM/s (Cadenas and Davies, 2000). Thus, a radiation dose of 1 Gy initially generates, at most, as much $^{\bullet}O_2^-$ as humans produce in around 20 s in the same volume, averaged over the cell. The comparison is even more marked considering the rate of production of $^{\bullet}O_2^-$ in mitochondria, which has been estimated to be $\sim$ 0.6 μM/s averaged over the mitochondrial volume (Cadenas and Davies, 2000).

In order to assess the possibility of which sites for free radical damage might function in a controlling/signaling pathway, it is useful to consider the possible general targets for radical reactions. The same logic is used to evaluate whether $ONOO^-$ (or its conjugate acid) reacts directly with a target or whether the effects occur via decomposition of $ONOO^-$ into radicals by either of the pathways shown in Figure 1. The decomposition of $ONOO^-$ at pH 7.4, 37 °C has a first-order rate constant ($=\ln 2/$(half-life)) of about 1 s^{-1} in the absence of CO_2. At physiological levels of CO_2 *in vivo* (e.g., in plasma), its half-life is $\sim$9 ms (rate constant $\sim$75 s^{-1}; Denicola *et al*., 1996). In Table 1, second-order rate constants are listed for reactions of some radicals and $ONOO^-$, with a range of potential physiological targets. The likelihood of significant reaction with a target can be assessed by comparing the product of rate constant and concentration with the first-order rate constants for decomposition and/or reaction with CO_2, as appropriate. Such calculations have been reported (e.g. Alvarez *et al*., 1999; Arteel *et al*., 1999). Obviously, the conclusions are subject to considerable uncertainty because it is difficult to extrapolate measurements of average concentrations to likely values in cellular organelles. Some recent studies specifically consider $ONOO^-$ reactions in mitochondria (e.g. Radi *et al*., 2002).

Particularly noteworthy are the high rate constants for reaction of $ONOO^-$ with some proteins containing metal-thiolate clusters, compared with reaction with low-molecular-weight thiols. Complexing thiols with metals, particularly iron and zinc, appears to change the redox properties of the thiol moiety, making it more susceptible to oxidation. Thus, the Fe^{2+}/cysteine complex reduces oxygen to $^{\bullet}O_2^-$ rapidly; zinc competes effectively for binding, but reduces $^{\bullet}O_2^-$ formation (Willson, 1977). Typical of iron/sulfur proteins is aconitase (Castro *et al*., 1994), which is also highly reactive toward $^{\bullet}O_2^-$ (Hausladen and Fridovich, 1994), releasing iron, and also toward $ONOO^-$ (Castro *et al*., 1994). Zinc-thiolate centers are also very reactive toward

Table 1 Approximate rate constants ($M^{-1}s^{-1}$) for reactions of some species involved in oxidative and nitrosative stress, pH 7.4, ~25°C[a]

	$^{\bullet}OH$	$^{\bullet}O_2^-$	$NO_2^{\bullet}$	$^{\bullet}CO_3^-$	$ONOO^-/ONOOH$
GSH	9×10^9	2×10^2	2×10^7	5×10^6	6×10^2
Cysteine	2×10^{10}		5×10^7	5×10^7	6×10^3
Ascorbate	1×10^9	5×10^4	4×10^7	$1\times10^{9\,b}$	5×10^1
Urate	7×10^9		2×10^7		5×10^{2c}
Tyrosine	1×10^{10}	$<1\times10^1$	3×10^5	5×10^7	
Tryptophan	1×10^{10}	$<2\times10^1$	$<5\times10^5$	7×10^8	4×10^{1c}
Methionine	9×10^9	<1		$1\times10^{8\,b}$	2×10^2
NADH	2×10^{10}	1×10^5	4×10^3	1×10^9	
Tetrahydrobiopterin	9×10^9		1×10^9	5×10^9	
Aconitase		3×10^7			1×10^5
Cytochrome *c* (Fe(II))	$>1\times10^{10}$	1×10^6			3×10^4
Mn-SOD	2×10^9				1×10^5
CO_2					3×10^4

[a]Most rate constants are from the NIST database (Ross *et al*., 1998) or references mentioned in the text (Radi *et al*., 2000; Ford *et al*., 2002a; Kirsch *et al*., 2002; Patel *et al*., 2002; Radi *et al*., 2002). [b]At pH 11.4. [c]At 37°C

$ONOO^-$, with a rate constant of $\sim3\times10^5\,M^{-1}s^{-1}$ measured in yeast alcohol dehydrogenase (Crow *et al*., 1995). The low-molecular-weight complex of zinc with 2,3-dimercaptopropanol reacted with $ONOO^-$ with a rate constant of $\sim4\times10^4\,M^{-1}s^{-1}$ (Crow, 2000). The importance of zinc-thiolate complexes as targets is that such a center stabilizes dimeric NOS, and $ONOO^-$ treatment releases zinc from the endothelial isoform (NOS-3), disrupting $NO^{\bullet}$ synthesis in favor of $^{\bullet}O_2^-$ generation (Zou *et al*., 2002). Tetrahydrobiopterin (H_4B) is an essential cofactor for all NOS isoforms and is extremely reactive toward $NO_2^{\bullet}$ and $^{\bullet}CO_3^-$ radicals (Patel *et al*., 2002) (Table 1). The biopterin radical formed on one-electron oxidation is likely to be repaired by ascorbate *in vivo* (Table 1). While both GSH and ascorbate are protective toward oxidation of H_4B by $NO_2^{\bullet}$, because the high concentrations of these antioxidants outweigh the lower reactivity toward $NO_2^{\bullet}$ compared with H_4B, thiol scavenging may not be protective overall because the thiyl radical $GS^{\bullet}$ oxidizes H_4B very rapidly (Patel *et al*., 2002). As with the zinc-thiolate complexes, H_4B is important in controlling the relative efficiencies of NOS as either $NO^{\bullet}$ or $ONOO^-$ generators (Stuehr *et al*., 2001). Depleting H_4B switches NOS to a 'peroxynitrite synthase' by stimulating more $^{\bullet}O_2^-$ formation. In turn, this is a true amplification mechanism because the more powerful oxidants thus produced ($NO_2^{\bullet}$, $^{\bullet}CO_3^-$, $^{\bullet}OH$) further deplete H_4B and drive a self-amplifying cascade.

In addition to zinc-thiolate complexes and H_4B as important targets in nitrosative stress, the regulatory role of $NO^{\bullet}$ and cytochrome *c* oxidase in extending the perfusion distance of oxygen (Brookes *et al*., 2002) is of note. Peroxynitrite reactions in mitochondria (Radi *et al*., 2002) and activation of stress response pathways (Klotz *et al*., 2002) have been recently reviewed. It was concluded (Radi *et al*., 2002) that cytochrome *c* oxidase (complex IV)-dependent respiration is not affected by $ONOO^-$, but release of mitochondrial cytochrome *c* following exposure to elevated ROS/RNS has been reported and linked to nitration of a critical tyrosine residue. One possible mechanism suggested by Radi *et al*. (2002) is $ONOO^-$-stimulated nitration of the tyrosine-34 site of mitochondrial Mn-SOD. Inactivating SOD by nitration increases the lifetime of $^{\bullet}O_2^-$ radicals and, hence, further $ONOO^-$ formation from $NO^{\bullet}$ in a 'positive feedback cycle'. Nitration of Mn-SOD is observed following exposure of cells to low doses of radiation as well, and this target may be of central importance in amplifying the radiation response as discussed below (Leach *et al*., 2002).

In conclusion, the involvement of nitrosative stress in modulating the function of NOS can be linked to reactions between reactive oxidants and both zinc-thiolate complexes and H_4B. In the wider context, other damage amplification reactions include the release of Fenton-catalytic iron from aconitase by $^{\bullet}O_2^-$. As discussed below, zinc-thiolate centers are also important in a number of redox-sensitive transcription factors, including those activated by ionizing radiation and that are involved in generating and amplifying cytoprotective responses.

Cellular mechanisms of ROS homeostasis

ROS free radicals and their reactive nonradical derivatives, such as H_2O_2, are present in all cells at very low concentrations established by a balance of generating and neutralizing processes. Disruption of this balance by adding oxidants (e.g. ionizing radiation) or antioxidants triggers redox signaling and induction of cellular protective mechanisms that re-establish the initial redox/free radical balance (Dröge, 2001).

As discussed above, most cellular ROS in nonphagocytic cells is produced by endogenous mitochondrial electron transport. Another important source of $^{\bullet}O_2^-$ is the plasma membrane NADPH oxidase, which has been extensively studied in phagocytic cells where it plays a prominent role in antimicrobial and tumoricidal oxidative burst. NADPH oxidase isoforms have also been described in nonphagocytic cells. These latter isoforms produce considerably less $^{\bullet}O_2^-$ than the enzyme found in macrophages and neutrophils and that generated by mitochondrial respiration. The roles of the nonphagocytic NADPH oxidase are not completely understood,

but appear to be tissue dependent (Dröge, 2001). For example, vascular smooth muscle cells appear to generate intracellular $^{\bullet}O_2^-$ in contrast to extracellular generation in endothelial cells, fibroblasts and neutrophils. NADPH oxidase is an FAD-requiring enzyme. Inhibition of $^{\bullet}O_2^-$ production by the FAD analog, diphenyliodinium, is often taken as evidence for NADPH oxidase-dependent $^{\bullet}O_2^-$ generation. However, other cellular sources of ROS/RNS are also FAD-dependent and sensitive to diphenyliodinium, for example, mitochondrial electron transport (Majander *et al*., 1994).

Cellular SOD converts the $^{\bullet}O_2^-$ anion to H_2O_2, which is then further reduced to water by catalases and peroxidases in the cytoplasm and in the mitochondria by an NADPH-dependent glutathione peroxidase. As discussed above, NO$^{\bullet}$ also modulates cellular ROS levels. When produced at sufficiently high levels by NOS, NO$^{\bullet}$ can react with $^{\bullet}O_2^-$ to form $ONOO^-$. $ONOO^-$ mostly rearranges to relatively innocuous nitrite/nitrate, converted to such by cytochrome *c* oxidase, or reacts with GSH to form GSNO. The latter represents a potential storage form of NO$^{\bullet}$. Thus, at modest NO$^{\bullet}$ levels, its reaction with and neutralization of $^{\bullet}O_2^-$ represents a cytoprotective mechanism (Wink *et al*., 1995; Beckman and Koppenol, 1996).

Other studies have suggested that endogenously produced NO$^{\bullet}$ protects cells by inhibiting cytochrome *c* oxidase, mitochondrial electron transport and thus endogenous $^{\bullet}O_2^-$ generation (e.g. Kanai *et al*., 2001; Paxinou *et al*., 2001; Sarkela *et al*., 2001; Elfering *et al*., 2002). However, recent careful measurements of concentration-response curves for mitochondrial respiration in brain slices bring into question the role of cytochrome *c* oxidase. At least in this tissue the amount of NO$^{\bullet}$ generated under physiological conditions with maximal stimulation of NOS isoform 1 (NOS-1) activity was found to be too low to inhibit mitochondrial respiration but sufficient to fully activate soluble guanylate cyclase (Bellamy *et al*., 2002).

Although the exact mechanism remains to be defined, other evidence also supports a mitochondria-dependent mechanism. Thus, endogenously generated NO$^{\bullet}$ protects cells against H_2O_2 exposure only when cells are actively respiring (Paxinou *et al*., 2001). The importance of NO$^{\bullet}$ in regulating cellular ROS levels is underlined by the recent identification of a Ca^{2+}-activated NOS isoform in the mitochondria of cells (Kanai *et al*., 2001; Elfering *et al*., 2002). Pharmacological inhibition of cellular NOS stimulates $^{\bullet}O_2^-$ production, and NO$^{\bullet}$ scavengers induce cellular oxidative stress (Niu *et al*., 1996; Goda *et al*., 1997; Janssen *et al*., 1998a, b).

When NO$^{\bullet}$ levels approach those of SOD, higher, cytotoxic levels of $ONOO^-$ are reached (Beckman and Koppenol, 1996). Cell-damaging effects are seen after treatment of cells with very high concentrations of NO$^{\bullet}$ donors or by expressing inducible isoforms of NOS that generate large amounts of NO$^{\bullet}$ (Szabo and Ohshima, 1997; Phoa and Epe, 2002).

Cellular mechanisms of NO$^{\bullet}$ homeostasis

In contrast to $^{\bullet}O_2^-$, which is mostly a by-product of inefficiency in mitochondrial electron transport, NO$^{\bullet}$ is generated by specific enzymes, the NO$^{\bullet}$ synthases (Stuehr, 1999). The activities of these enzymes are regulated at multiple transcriptional and post-transcriptional levels. NO$^{\bullet}$ is released during the catalytic conversion of arginine to citrulline in a complicated mechanism involving multiple cofactors, including H_4B, NADPH, FMN and an Fe–S center. Three types of NOS have been described with the basic catalytic mechanism conserved among the three (Stuehr, 1999). Both the neuronal synthase (NOS-1) and endothelial synthase (NOS-3) enzymes are named from the tissues in which they were first discovered but are constitutively expressed in many cell types. Although considered to be constitutively expressed, NOS-1 mRNA transcription is under a high level of control. The NOS-1 gene is made up of 27 exons and encodes for several different isoforms with molecular sizes ranging from 150 to 165 kDa. A specific isoform of NOS-1 has been identified in the mitochondria of liver and cardiomyocytes (Kanai *et al*., 2001; Elfering *et al*., 2002), and its mRNA transcript has been identified in most if not all tissues examined (Elfering *et al*., 2002). The mitochondrial NOS-1 is myristoylated in contrast to other NOS-1 isoforms.

The NOS isoforms first identified in endothelial cells, NOS-3, are also myristoylated proteins of 130–135 kDa that, like NOS-1, require dimer formation for catalytic activity. NOS-3 is primarily located in the plasma membrane of cells. The requirement for dimer formation has been used to develop, for both NOS-1 and NOS-3, mutants that when expressed inhibit endogenous NOS activity (Lee *et al*., 1995; Phung and Black, 1999).

NOS-1 and NOS-3 produce relatively low amounts of NO$^{\bullet}$ compared with the third major isoform, inducible NOS (iNOS or NOS-2). NOS-2 activity is normally associated with macrophages but can be transcriptionally activated by different cytokines (e.g. interferon-γ) in a number of cell types (Janssen *et al*., 1998a; Freeman and MacNaughton, 2000; Yoo *et al*., 2000). High levels of NO$^{\bullet}$ and $OONO^-$ formation at inflammatory sites are associated with activation of NOS-2.

The expression of NOS-2 is the predominant factor regulating its cellular activity. This contrasts with the number of post-translational regulatory mechanisms for NOS-1 and NOS-3. The catalytic activities of both of these isoforms are tuned to changes in cellular $[Ca^{2+}]$ by a Ca^{2+}/calmodulin-dependent mechanism. Since NOS-2 tightly binds calmodulin, its activity is largely independent of $[Ca^{2+}]$. As will be discussed below, a change in cytoplasmic $[Ca^{2+}]$ is one process by which a signal initiated by ROS formation can be converted/translated into NO$^{\bullet}$-dependent signaling. Several recent studies have shown that NOS-1 and NOS-3 are also regulated by phosphorylation (Michel *et al*., 1993; Cordelier *et al*., 1999; Elfering *et al*., 2002; Leach *et al*., 2002). In some tissues, NOS-1 associates with adaptor proteins and

small monomeric G proteins specific for the tissue. Thus, Dexras1, a G protein found predominantly in the brain, forms a ternary complex with NOS-1 and an adaptor protein (CAPON) and is activated when NOS-1 is activated (Fang *et al*., 2000).

Breakdown of $NO^{\bullet}$ has been until recently thought to be due to the reaction of $NO^{\bullet}$ with oxyhemoglobin or oxymyoglobin producing nitrate and methemoglobin and metmyoglobin. The former reaction has been brought into question (Gow *et al*., 1999), and it appears unlikely that the oxymyoglobin reaction is relevant at $NO^{\bullet}$ concentrations obtained under normal noninflammatory conditions. Recent studies (Pearce *et al*., 2002) suggest that a reaction catalyzed by cytochrome *c* oxidase to produce nitrite is a potential catabolic pathway for $NO^{\bullet}$ at physiological levels of $NO^{\bullet}$ in mitochondria-rich tissues. In cells of low mitochondrial content, such as endothelial cells (and tumor cells?), a net result would be to facilitate diffusion of $NO^{\bullet}$ into the vasculature, minimizing its consumption in the tissue and thus facilitating activation of sGC and other $NO^{\bullet}$-activatable targets (Pearce *et al*., 2002).

$NO^{\bullet}$ can also be produced by routes not involving NOS. In addition to the metal-catalyzed breakdown of *S*-nitrosothiols described above, production of $NO^{\bullet}$ in tissues subject to ischemia/reperfusion cycles can be demonstrated, involving reduction of nitrate/nitrite catalyzed by xanthine oxidase (Millar *et al*., 1998; Li *et al*., 2001).

GSH, subcellular compartmentalization and the redox status of cells

A key feature of eucaryotic cells in considering redox sensors and homeostasis is cellular compartmentalization. A measure of the redox status of different cellular compartments is the GSH : GSSG ratio. Under normal physiological conditions, the GSH : GSSG ratios of the different cellular compartments are: cytoplasm (>100), endoplasmic reticulum (≈ 1–2), mitochondria (≈ 5–10) (Hwang *et al*., 1992; Morales *et al*., 1998). There is evidence that the nuclear GSH : GSSG ratio can be different from that of the cytoplasm (Voehringer *et al*., 1998). Thus, in considering the response of cells to oxidative or nitrosative events, such as produced by radiation, the localization of sensing mechanisms in cellular compartments of differing reductive environments requires consideration.

A number of studies have shown that cells are radiosensitized when total cellular GSH levels drop below 20% (e.g. Meister, 1994 and references therein). Cells depleted of GSH continue to divide at normal rates and only when challenged by an additional oxidative stress such as exposure to ionizing radiation do they commence dying. One interpretation is that radiosensitization is only observed when GSH levels are reduced sufficiently to be unable to counter the amount of ROS produced after radiation. However, even with this extensive depletion, total cellular GSH concentration remains about 1 mM and only small amounts of ROS relative to that produced by metabolism are produced as a direct consequence of the radiation-induced ionization events (Ward, 1994). As a result, this appears to be an unlikely explanation. An alternative possibility follows from an observation by Meister (1994) that mitochondria do not synthesize their own GSH but depend on high-affinity transport and cytoplasmic GSH synthesis. Only when cytoplasmic GSH levels drop below 20%, do mitochondrial GSH levels decrease. The net effect of this decrease is to reduce mitochondrial GSH peroxidase activity, the primary mechanism of H_2O_2 detoxification in mitochondria, and thus increase endogenous levels of metabolically produced H_2O_2. Since we have shown that radiation also stimulates mitochondrial ROS/RNS generation (Leach *et al*., 2001), the combined increase due to activation of ROS/RNS generation and the elevated ROS generation due to endogenous metabolism may be sufficient to overwhelm the mitochondrial redox homeostasis properties of GSH-depleted cells. Additional evidence for this proposal will come from experiments where mitochondrial GSH is specifically targeted and in studies using ρ^{o} cells that are deficient in mitochondrial electron transport (King and Attardi, 1996; Leach *et al*., 2001).

An important aspect of GSH metabolism that has not been considered in radiation biology is the GSNO pool. Tissue concentrations of GSNO are in the μM range compared to the nM levels for $NO^{\bullet}$ (Gaston, 1999) and thus GSNO (and other *S*-nitrosothiols) represents a potential reservoir and buffer of cellular NO. As discussed above, GSNO formation may be linked to radical damage from ROS/RNS, since the repair of this damage involves thiols, the production of thiyl radicals and the probable diffusion-controlled reaction of thiyl radicals with $NO^{\bullet}$. Intracellular levels of GSNO are also tightly coupled with NOS activity and various GSNO-metabolizing enzymes. Thus, breakdown of GSNO by thioredoxin reductase or glutathione peroxidase results in the release of $NO^{\bullet}$ (Gaston, 1999). Increases of 10-fold in cell *S*-nitrosothiols are indicative of nitrosative stress and are associated with induction of apoptosis (Eu *et al*., 2000).

Ionizing radiation and redox-sensitive signaling

Ward (1994) originally raised the question of how cells sense and react to the relatively low amounts of ROS produced by clinically relevant radiation doses with background ROS levels 100–1000-fold higher. This differential may be even greater with tumor tissues that in general show much higher rates of ROS generation than normal cells (e.g. Szatrowski and Nathan, 1991). Recent studies measuring ROS/RNS generation in irradiated cells have provided possible answers.

ROS/RNS production induced by radiation has been measured with fluorescent dyes (Clutton *et al*., 1996; Narayanan *et al*., 1997; Morales *et al*., 1998). These

studies (keeping in mind the caveats about fluorescent dyes discussed above) demonstrated a relatively early response above baseline metabolic levels at both high and low linear energy transfer radiations and within 30 min of radiation exposure. Measurements of ROS/RNS generated after irradiating cells reveal that much more ROS/RNS is produced than can be accounted for by both primary and secondary products of the ionization events, suggesting an amplification mechanism. Inhibition of the enhanced dye fluorescence with an FAD analog binding inhibitor suggested that alpha-particle radiation stimulated NADPH oxidase activity (Narayanan *et al*., 1997).

More recent studies used digitized fluorescence microscopy to measure changes in ROS/RNS-sensitive dye fluorescence at the single cell level and a combined pharmacological and genetic approach to establish more definitively the source of the ROS/RNS generated after a radiation exposure (Leach *et al*., 2001, 2002). A ^{90}Sr eye applicator was mounted on an inverted microscope to permit fluorescence image collection within seconds of commencing radiation exposure. Radiation-stimulated ROS/RNS generation occurred within seconds of starting radiation treatment (1–10 Gy) and persisted for 2–5 min postirradiation in several epithelial cell lines (Leach *et al*., 2001). The amount of ROS/RNS generated per cell was relatively constant over this radiation dose range, but increasing numbers of cells responded with enhanced ROS/RNS generation with dose escalation. These results are consistent with a threshold, possibly all-or-nothing, response. When plotted as a traditional semilog plot of responding cells versus radiation dose, a straight line extrapolating to 1 was obtained – consistent with a single target.

Cells depleted of mitochondrial DNA and thus deficient in mitochondrial electron transport did not demonstrate a radiation-stimulated increase in ROS/RNS. Furthermore, chelation of intracellular Ca^{2+} or incubating cells with cyclosporin A or bongkrekic acid also inhibited radiation-induced ROS/RNS. The latter two drugs target components of the pore composing the mitochondrial ion channel responsible for the Ca^{2+}-sensitive mitochondrial permeability transition (Ichas and Mazat, 1998). Based on these studies, it was proposed that radiation activated a *reversible* form of the permeability transition that propagated from one mitochondrion to adjacent mitochondria by a membrane depolarization Ca^{2+}-release mechanism with consequential release of ROS. Additional evidence for this proposal came from measuring radiation-induced mitochondrial membrane depolarization and enhanced permeability of the mitochondrial membrane to the fluorescent small molecule, calcein. This model fits with previous findings from other investigators demonstrating such propagating signals within a cell's mitochondrial pool and as a mechanism involved in Ca^{2+} homeostasis and cellular redox modulation (Bernardi and Petronelli, 1996; Vercesi *et al*., 1997; Ichas and Mazat, 1998; Romashko *et al*., 1998; Zorov *et al*., 2000). Specifically, Zorov *et al*. (2000) proposed that ROS-induced ROS release accompanies the mitochondrial permeability transition and provides a 'self-amplifying' mechanism by which redox signals can be transmitted throughout the cell.

At present, the mitochondrial sensor of the oxidative/ionization event that triggers the mitochondrial permeability transition is not known. A number of studies using sulfhydryl-reacting agents have emphasized the importance of mitochondrial protein thiols in regulating the permeability transition (Bernardi and Petronelli, 1996; Vercesi *et al*., 1997; Crompton, 1999). Zorov *et al*. (2000) have evidence for a critical role for mitochondrial thiols in the initiation and propagation of the mitochondrial permeability transition during ROS-induced ROS release in cardiac myocytes. A recent study (Pearce *et al*., 2001) demonstrated that radiation at relatively high doses inhibited respiratory complexes I (NADH dehoydrogenase) and III (cytochrome *c* reductase) but not complexes II (succinate dehydrogenase), IV (cytochrome *c* oxidase) and V (ATP synthase). This is not, however, evidence for their roles as sensors, since inhibition was only consistently observed hours after irradiation.

Two features of this model for a redox response mechanism to radiation warrant further comment. Firstly, the model follows traditional radiobiological theory that emphasizes target volume. After the nucleus, the largest cellular target volume is the mitochondria volume representing between 4 and 30% of total cell volume depending on cell type. Since the signal can propagate from one mitochondrion to another, the permeability transition of only one mitochondrion is required for propagation and thus amplification of the initial oxidative event. Release of Ca^{2+} initiates signal propagation to adjacent mitochondria and potentially throughout the mitochondrial pool of a cell. This fits with the apparent all-or-nothing response that is observed. The mitochondrion is also the major source of cellular ROS. A number of past studies have also provided indirect evidence for a mitochondrial role in the cellular response to ionizing radiation (e.g. Gudz *et al*., 1994 and references therein).

Secondly, the radiation-induced burst of ROS/RNS generation is transient. This has important implications in terms of the final cellular response. If the radiation-stimulated ROS/RNS generation is not transient, one would predict on the basis of a continuous elevated production of ROS/RNS that the final cellular response would be cell death. On the other hand, transient responses suggest that regulatory, possibly cytoprotective, components are being engaged. A linkage between the radiation-stimulated ROS/RNS generation and an important cytoprotective response has been established as described below.

Subsequent studies demonstrated that a major contributor to the radiation-induced oxidation of $DCFH_2$ fluorescence signal was probably $ONOO^-$, observed after activation of NOS (Leach *et al*., 2002). Molecular analyses with expression of wild-type and a dominant negative mutant of NOS-1 and measurements of NOS catalytic activity with the arginine–citrulline assay confirmed that radiation transiently activated a Ca^{2+}-

dependent NOS-1 in Chinese hamster ovary cells within the time frame observed for ROS/RNS generation. Radiation stimulated tyrosine nitration of a number of proteins, including MnSOD, by a mechanism inhibited by expression of the dominant negative mutant of NOS-1, consistent with formation of $ONOO^-$. These findings are also consistent with those from an earlier study with cardiac myocytes, in which a photochemically induced mitochondrial ROS release was measured with $DCFH_2$ (Zorov *et al*., 2000). $NO^\bullet$ was generated as measured with DAF-2, but apparently at significantly lower rates relative to the ROS burst.

The radiation-induced MAPK1/2 activation thought to be a cytoprotective response to ionizing radiation and observed by several investigators (reviewed in Schmidt-Ullrich *et al*., 2000) was found to be a consequence of the radiation-stimulated ROS/RNS generation (Leach *et al*., 2001, 2002). The radiation-induced MAPK1/2 activation was dependent on NOS-1 activity, as shown by both genetic and pharmacological methods, but the actual mechanism by which this occurs remains undefined. MAPK activity is regulated positively by tyrosine phosphorylation via downstream signaling from growth factor receptors to RAS (a GTP/GDP exchange protein), RAF kinase and MEK1/2 dual function kinases that phosphorylate MAPK1/2 on threonine-202 and tyrosine-204. MAPK is negatively regulated by the activity of different protein tyrosine phosphatases that not only can act on MAPK but also on upstream components including growth factor receptors and RAF-1 kinase. The activities of all these proteins are potentially modulated by ROS/RNS, but preliminary studies suggest that S-nitrosylation and inhibition of a protein tyrosine phosphatase may be critical (see below).

Biological ROS/RNS sensors: technical issues

ROS/RNS concentrations and reaction rates were discussed in evaluating the relative significance of the different pathways in forming reactive oxidants from $^\bullet O_2^-$ and $NO^\bullet$ radicals (Figure 1). These same considerations apply in identifying physiologically relevant ROS/RNS sensors and signal transduction pathways activated by ROS/RNS. Of most importance for reasons discussed above, measurements of intracellular ROS/RNS concentrations and identification of the specific ROS/RNS involved are inherently difficult, if not impossible, with intact cells and presently available experimental techniques. The problem may be compounded by difficult-to-measure localized subcellular changes in ROS/RNS of sufficient magnitude to be of biological significance.

Much of the work on defining the roles of ROS or RNS as redox second messengers has relied on the use of relatively high concentrations of oxidants, usually H_2O_2, or $NO^\bullet$ donors, as initiators. The difficulties in using such an approach can be seen in studies on protein tyrosine phosphatases (e.g. Lee *et al*., 1998; Meng *et al*., 2002). Addition of high concentrations of H_2O_2 to cells clearly oxidizes the active site cysteine of the protein tyrosine phosphatase to sulfenic acid and inhibits catalytic activity. Whether this has anything to do with the *in vivo* situation, where much less H_2O_2 is metabolically generated, is less clear. In some experimental settings, catalase has been added as proof that H_2O_2 is the oxidizing agent. The significance of this control is uncertain since the added catalase is extracellular, whereas the action of H_2O_2 is presumably intracellular, although H_2O_2 gradients across biomembranes can be estimated (Antunes and Cadenas, 2000). Even elevation of intracellular catalase activity by genetic means is not definitive proof. Overexpression of catalase may simply shift the overall redox balance of the cell. One can also explain the H_2O_2-induced inhibition of protein tyrosine phosphatases by an RNS-based mechanism. In this scenario, H_2O_2 treatment or other oxidative stress stimulates the release of Ca^{2+} from intracellular stores with subsequent activation of Ca^{2+}-dependent NOS activity (Todd and Mikkelsen, 1994; Srivastava *et al*., 1999; Droge, 2001; Leach *et al*., 2002). $NO^\bullet$ or $ONOO^-$ also oxidizes protein cysteine to sulfenic acid (e.g. Stamler and Hausladen, 1998). S-thiolation is another mechanism that needs consideration (e.g., Eaton *et al*., 2002).

Besides the potential confusion over what ROS or RNS is involved, the biological response can vary with ROS and RNS. This is to be expected in signal transduction pathways with multiple inputs of variable oxidative or nitrosative sensitivities. The biphasic response of the transcription factor NF-κB to different $NO^\bullet$ concentrations exemplifies this (Sheffler *et al*., 1995).

Transcription factors as ROS/RNS sensors

ROS sensors in eucaryotic cells remain mostly undefined, but clues to their molecular nature come from what is known about bacterial ROS sensors. The best described are OxyR and SoxR as sensors for H_2O_2 and $^\bullet O_2^-$, respectively. The SoxR protein regulates the synthesis of several proteins at the sox locus involved in the protective response to $^\bullet O_2^-$. SoxR is a homodimer with two 2Fe–2S clusters, one in each subunit, that under normal conditions exist in the reduced state (Bauer *et al*., 1999). Fe oxidation appears critical for transcription initiation, but the underlying mechanisms remain mostly undefined (Hidalgo *et al*., 1997). Thus, $^\bullet O_2^-$ can oxidize SoxR but only at supraphysiological levels. Although H_2O_2 can oxidize SoxR Fe, it does so without stimulating biological activity. $NO^\bullet$ does not oxidize SoxR but $NO^\bullet$ modulates biological activity *in vivo* (Nunoshiba *et al*., 1995). As pointed out by Marshall *et al*. (2000), no eucaryotic homologs of SoxR have been described nor have eucaryotic transcription factors with redox-sensitive transition metals been identified.

H_2O_2 activates OxyR by oxidation of cysteine-199 to sulfenic acids which subsequently forms an intramolecular disulfide bond with cysteine-208 (Zheng *et al*., 1998; Zheng and Storz, 2000). This conversion of reduced to oxidized OxyR can also be achieved by changing the intracellular thiol : disulfide ratio. *S*-nitrosothiols activate the OxyR protein possibly by S-nitrosylation of cysteine-199 (Marshall *et al*., 2000; Zheng and Storz, 2000).

Eucaryotic cells also have redox-sensitive transcription factors that potentially act as ROS sensors. Probably, the best studied are AP-1 and NF-κB. The DNA-binding activities of both are responsive to the cellular thiol–disulfide ratio due to single cysteines in the DNA-binding domains of these transcription factors (e.g. Abate *et al*., 1990; Matthews *et al*., 1992). Oxidation of these cysteines blocks binding of the transcription factors to their respective consensus DNA sequences. Not unexpectedly, the sequence-specific binding to DNA is enhanced by reducing conditions. Interestingly, a dual-function protein (REF-1) functions as a reducing agent for AP-1, but it also has another domain with endonuclease activity involved in DNA repair (Xanthoudakis *et al*., 1992). Thioredoxin restores transcriptional activity of AP-1 by binding to AP-1 and by complexing with and maintaining REF-1 in its reduced state.

The redox- and radio-responsive transcription factor NF-κB is characterized by the redox-sensitive cysteine-62 in the DNA-binding domain of the P50 subunit. As with AP-1, REF-1, chemical thiol-reducing agents and thioredoxin can restore DNA-binding activity of NF-κB (Matthews *et al*., 1992; Marshall *et al*., 2000).

Although DNA binding of these transcription factors is favored by reducing conditions, oxidative stress, including ionizing radiation, usually activates both AP-1 and NF-κB. This apparent dichotomy is the result of differential redox regulation in the cytoplasm and nucleus, combined with other signaling pathways promoted by oxidative conditions in the cytoplasm that initiate the activation of NF-κB and AP-1 prior to translocation into the nucleus. Using an elegant approach of protein thiol modification and liquid chromatography–mass spectroscopy analysis, Nishi *et al*. (2002) determined the redox state of different cysteines of the P65 and P50 subunits of NF-κB. The cysteines of both proteins were equivalently reduced in either the cytoplasm or nucleus with one exception. Cysteine-62 in the DNA-binding domain of P50 was highly oxidized in the cytoplasm and reduced in the nucleus. The differential redox state of cysteine-62 was maintained in part by nuclear REF-1. The reduced state of P50 cysteine-62 is critical for transcriptional activity (Matthews *et al*., 1992; Marshall *et al*., 2000).

$NO^{\bullet}$ donors in a similar fashion can sensitize cells to apoptosis by inhibiting NF-κB by different mechanisms depending on cell type (Marshall and Stamler, 2002). In A549 cells, $NO^{\bullet}$ inhibits NF-κB DNA binding by S-nitrosylation of the P50 subunit. In Jurkat T cells, inhibition occurs in the cytoplasm prior to degradation of IκB.

Examples of signaling pathways activated by oxidative and nitrosative stresses include the release of intracellular Ca^{2+} involved in activation of both NF-κB and AP-1 and observed after treating cells with either H_2O_2 or with ionizing radiation (Todd and Mikkelsen, 1994; Dröge, 2001). The proteolytic degradation of IκB, the endogenous inhibitor of NF-κB, is also triggered by oxidative stress, although the mechanism by which this occurs may differ depending on the type of cell and stress (Karin, 1999). An alternative mechanism for NF-κB activation involves the phosphorylation of tyrosine-42 of IκB but without its proteolytic degradation. Cells that express IκB mutated at tyrosine-42 are more radiosensitive compared with parental and vector-transfected cells (Miyakoshi and Yagi, 2000). Phosphorylation of tyrosine-42 may be sensitive to oxidative stress since protein tyrosine phosphatase inhibitors enhance tyrosine phosphorylation of IκB with resultant NF-κB activation (Imbert *et al*., 1996), and protein tyrosine phosphatases are characterized by a redox-sensitive active site cysteine, responsive to oxidative and nitrosative stresses (e.g. Lee *et al*., 1998; Meng *et al*., 2002). Other stress-activated serine/threonine protein kinases, depending on cell type and type of stress, may also modulate NF-κB and AP-1 transcriptional activities (e.g. Stephenson *et al*., 1994; Wilhelm *et al*., 1997; Norris and Baldwin, 1999; Marshall *et al*., 2000; Miyakoshi and Yagi, 2000; Park *et al*., 2000; Droge, 2001; Howe *et al*., 2002).

Other transcription factors of radiobiological interest whose activities are sensitive to ROS/RNS include those with a zinc-finger (zinc-thiolate cluster) in the DNA-binding domain (e.g. EGR-1 and SP-1; Ahmed *et al*., 1997; Wang *et al*., 1999; Raju *et al*., 2000). Both ROS and RNS inhibit DNA binding of zinc-finger transcription factors by stimulating the release of the metal ion. However, there appears to be an important difference between the ROS and RNS mechanisms. Using the vitamin D receptor (VDR) and retinoid X receptor (RXR) as model zinc-finger transcription factors, Kroncke *et al*. (2002) showed that the zinc-fingers of VDR and RXR could be repaired after nitrosative but not oxidative stresses. This suggests that RNSs are not just inhibitors but regulators of zinc-finger transcription factor activities.

The tumor suppressor protein, TP53, is activated not only by radiation but also by treatment of cells with $NO^{\bullet}$ donors (Wang *et al*., 2002; Brune *et al*., 2001; Schonhoff *et al*., 2002). $NO^{\bullet}$ donors appear to act by two different mechanisms. *In vitro* studies show that in the short term, S-nitrosylation of the TP53 negative regulator Hdm2, inhibits its interaction with TP53. *In vivo* this disrupts TP53 ubiquitination and proteolysis, thereby increasing cellular TP53 levels (Schonhoff *et al*., 2002). $NO^{\bullet}$ treatment also down-regulates Mdm2 (mouse equivalent to Hdm2) expression, resulting in a corresponding drop in TP53 ubiquitination (Wang *et al*., 2002). It is not known whether $NO^{\bullet}$ has a significant role in the radiation-stimulated TP53 expression.

Effects of ROS/RNS on other zinc and iron proteins

Except for atypical isoforms, protein kinase C is activated either by binding the second messenger, diacylglycerol, or by oxidation facilitated by retinols that convert the protein to an active form (Korichneva *et al*., 2002 and references therein). The diacylglycerol/retinal-binding domain is contained within the twin cysteine-rich domains of six conserved cysteines and two histidines that form a tetrahedral coordinate complex with two zinc atoms. Treatment with either diacylglycerol or hydrogen peroxide induces the release of zinc, in turn inducing a reversible conformational change activating the kinase. The authors describe zinc as being a 'linchpin' that coordinates protein conformational changes in response to specific signals. These results have important implications for understanding the mechanism of oxidative/nitrosative modulation of other zinc-finger regulatory proteins, for example, RAF-1 kinase.

Calcineurin (protein phosphatase 2B) is activated by changes in cytosolic Ca^{2+} mediated by calmodulin. ROS, including hydrogen peroxide and $^{\bullet}O_2^-$ as well as certain sulfhydryl-reacting agents, inhibit calcineurin activity. Calcineurin – like some other serine/threonine protein phosphatases, PP1, PP2A, and purple acid phosphatase – contains a binuclear (Fe and Zn) metal center necessary for catalysis. Recent studies indicate that the native and redox-sensitive enzyme is the ferrous (Fe^{2+}–Zn^{2+}) form (Namgaladze *et al*., 2002). Superoxide radical at nanomolar concentrations orders of magnitude lower than hydrogen peroxide inactivates calcineurin in a Ca^{2+}/calmodulin-dependent mechanism that involves oxidation to a redox-insensitive Fe^{3+}–Zn^{2+} form. NO$^{\bullet}$ blocks the effect of $^{\bullet}O_2^-$. Thus, the activity of this key enzyme involved in diverse aspects of cell growth (as well as other protein serine/threonine phosphatases) responds to changes in cellular Ca^{2+}, NO$^{\bullet}$ and $^{\bullet}O_2^-$ concentrations.

Appropriate regulation of cellular iron levels is essential for providing cells with the necessary iron for metabolic needs (e.g. heme and nonheme iron proteins), while at the same time minimizing iron-catalyzed toxic-free radical formation. Iron regulatory proteins (IRPs) 1 and 2 are key components of iron homeostasis. When cellular iron levels are high, IRP-1 forms a (4Fe–4S) cluster, exhibits aconitase enzymatic activity and is no longer a regulator of ferritin or transferrin receptor expression. NO$^{\bullet}$ and, at much lower concentrations, $ONOO^-$ activate IRP-1 by inhibiting aconitase activity and removing iron from the Fe–S cluster (Cairo *et al*., 2002). SOD blunts the effects of NO$^{\bullet}$, suggesting that $ONOO^-$ is the critical species for disassembling the Fe–S cluster. IRP-2 does not contain an Fe–S cluster and, at low cellular iron levels, acts, as does IRP-1, as a post-transcriptional regulator of ferritin and transferrin receptor expression. IRP-2 is consistently inactivated by $ONOO^-$, probably by oxidizing protein cysteine and proteolysis (Kim and Ponka, 2000). The apparent dichotomy in the effects of $ONOO^-$ on IRP-1 and -2 activities is not understood but may provide clues as to the role of NO$^{\bullet}$ in regulating cellular iron levels.

NO$^{\bullet}$ also modulates zinc-dependent signal transduction pathways in its role as a regulator of zinc homeostasis. Essentially, all cellular zinc is protein bound. As a consequence, labile Zn^{2+} levels are dynamically maintained by the apposing pathways of NO$^{\bullet}$-stimulated release from metallothionein and sequestration by the apoprotein, thionein (Maret *et al*., 1999; St Croix *et al*., 2002). Thionein is a cysteine-rich protein that binds up to seven zinc atoms per protein molecule as zinc-thiolate clusters. Zinc is released from metallothionein by $^{\bullet}$NO-catalyzed S-nitrosylation. Thionein has been shown to extract zinc from glyceraldehyde 3-phosphodehydrogenase with a corresponding increase in enzyme activity and from the transcription factors, SP-1 and TF-IIIA, with resulting changes in their transcriptional activities (Zeng *et al*., 1991a, b; Maret *et al*., 1999). Thus, the ratio of metallothionein to thionein regulated by $^{\bullet}$NO can determine cellular zinc levels and the activities of a number of zinc-dependent signal transduction pathways (St Croix *et al*., 2002).

S-Glutathiolation modulation of protein function

S-glutathiolation of proteins links redox modulation of protein function directly with the GSH : GSSG ratio of a cell. S-glutathiolation is not that well studied but provides a mechanism for readily reversible oxidative modification of protein SH and represents an intermediate in reduction of protein sulfenic acid or S-nitrosylated Cys to SH. Besides several house-keeping proteins, a number of transcription factors and regulatory proteins have been shown to be S-glutathiolated under conditions of oxidative or nitrosative stress (Padgett and Whorton, 1998; Klatt and Lamas, 2000). Both c-JUN and NF-κB transcription factor DNA binding is redox modulated by specific S-glutathiolation (Pineda-Molina and Lamas, 2001). Recent technical strides in labeling cellular GSH and isolating proteins potentially S-glutathiolated should accelerate research in this area (e.g. Padgett and Whorton, 1998).

NO$^{\bullet}$ activation of soluble guanylate cyclase and activation of protein kinase G

At the low [NO$^{\bullet}$] produced by the NOS-1 and NOS-3 isoforms, activation of the heme-containing soluble guanylate cyclase and consequently PKG represents a major cellular function of NO$^{\bullet}$ (Francis and Corbin, 1999). Other heme proteins also bind NO$^{\bullet}$ and, in the case of cytochrome *c* oxidase, may be of importance in regulating cellular respiration as discussed above.

PKG are classified into two types encoded by different genes (Francis and Corbin, 1999). Two type I isoforms (α and β) arise by alternative splicing from a single gene and differ by 100 amino acids at their amino-terminal autoinhibitory domains but retain the same

cGMP binding and catalytic domains. PKG-I isoforms are expressed in diverse cell types. PKG-II is highly expressed in intestinal microvilli and, in contrast to soluble PKG-I, is associated with cellular membranes.

The *in vitro* study of PKG is hampered by its rapid downregulation in most cell types when grown in culture (Chiche *et al*., 1998; Komalavilas *et al*., 1999). Thus, the role of PKG in cellular growth regulation has relied on the use of primary cultures, transfection to overexpress PKG or the use of pharmacological activators and inhibitors of PKG or sGC. Modulation of PKG activity by these methods has revealed significant roles for the NO•-activated PKG-dependent signal transduction pathway in cell growth regulation (Cordelier *et al*., 1997; Chiche *et al*., 1998; Kim *et al*., 1999; Komalavilas *et al*., 1999; Gu *et al*., 2000). In many cases, this appears to be due to the intersection of PKG signaling with the MAPK and stress-activated kinase-dependent signal transduction pathways (Lander *et al*., 1996; Suhasini *et al*., 1998; Go *et al*., 1999; Komalavilas *et al*., 1999; Browning *et al*., 2000) and expression of the cyclin-dependent kinase inhibitor $P21^{Waf1/Cip1}$ (e.g. Gu *et al*., 2000). PKG has also been shown to modulate the activity of radio-responsive transcription factors (AP-1, CREB, TP53) and the expression of several genes whose expression is also stimulated by radiation, including JUN-B, c-FOS, MKP-1, cyclooxygenase-2, tumor necrosis factor-alpha and $P21^{Waf1/Cip1}$ (Haby *et al*., 1994; Gudi *et al*., 1996; Sciorati *et al*., 1997; Begum *et al*., 1998; Collins and Uhler, 1999; Gertzberg *et al*., 2000; Gu *et al*., 2000; Brune *et al*., 2001; Pfeilschifter *et al*., 2001).

Protein tyrosine-nitration

A footprint of $ONOO^-$ formation is tyrosine nitration of proteins. Formation of protein 3-nitrotyrosine has been detected in a number of disease states, but the functions of this protein modification remain unclear. Although there is some suggestion of reversibility, there is no convincing molecular evidence for a eucaryotic denitrase (Gow *et al*., 1996; Kamisaki *et al*., 1998). There appears to be some selectivity in the nitration, since not all proteins in a tissue under analysis are nitrated. Souza *et al*. (1999) carefully analysed nitration of three purified proteins under defined conditions and have determined some of the factors governing selectivity of nitration. Their evidence suggested that exposure of tyrosine at the surface of the protein, its location on a loop structure and an adjacent negative charge were some of the determining factors.

Not considered in this *in vitro* analysis but also of importance is subcellular location of the protein relative to the source of the nitrating agent. Thus, a prominent tyrosine-nitrated protein both in rejected human kidney allografts and in tissue culture cells after radiation is the mitochondrial protein Mn-SOD (MacMillan-Crow *et al*., 1996; Leach *et al*., 2002). In the case of rejected human allograft tissue, this nitration inhibits mitochondrial Mn-SOD enzymatic activity. As discussed above, inactivation of Mn-SOD increases the lifetime of $^{\bullet}O_2^-$ and hence formation of $ONOO^-$ from NO• by a 'positive feedback cycle' (Radi *et al*., 2002). Tyrosine nitration of Mn-SOD was also observed after exposing cells to ionizing radiation. A similar significant inhibition in enzymatic activity was not detected, possibly because only a small fraction of a very abundant cellular protein was nitrated (Leach *et al*., 2002). Nonetheless, if enzyme inhibition is limited to a single mitochondrion and thus not detectable by standard assay, significant biological effects can still accrue. This is because of potential intermitochondrial propagation of an oxidative event from one mitochondrion to adjacent mitochondria via a reversible permeability transition (e.g. Ichas and Mazat, 1998; Zorov *et al*., 2000; Leach *et al*., 2001, 2002). Thus, a potentially lethal oxidative event can be limited to a single mitochondrion among hundreds within a cell while at the same time initiating reversible cellular signaling pathways.

Several other biological effects have been attributed to tyrosine nitration. Tyrosine nitration has been shown to inhibit tyrosine hydroxylase activity under conditions of oxidative stress induced with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a model for Parkinson's disease (Ara *et al*., 1998). Addition of $ONOO^-$ has also been reported to induce tyrosine nitration and activation of P38, JNK and MAPK and block the interaction of the p85 regulatory subunit of phosphatidylinositol 3-kinase with the catalytic subunit (Hellberg *et al*., 1998; Schieke *et al*., 1999). There is recent evidence that NO• donors promote translocation and activation of PKCε via a mechanism of $ONOO^-$-mediated tyrosine nitration of PKCε, disrupting its interaction with RACK2 (Balafanova *et al*., 2002). The biological significance of many of these findings awaits the demonstration of reversibility (and the mechanism thereof) and quantitative evaluation of the degree of inhibition under physiological and pathophysiological endogenous conditions of $ONOO^-$ generation. It may be, as previously suggested, that tyrosine nitration marks the protein for proteolytic degradation (Gow *et al*., 1996; Ischiropoulos, 1998; Souza *et al*., 2000).

Protein S-nitrosylation

Recent studies have also established S-nitrosylation of protein cysteine as a major mechanism of NO• regulatory signaling (reviewed in Stamler *et al*., 1997; Broillet, 1999; Hess *et al*., 2001). Protein S-nitrosylation is linked to the activity of the different NOS isoforms depending on the cell type (Gow *et al*., 2002). In macrophages, induction of NOS-2 expression with cytokines enhances overall protein S-nitrosylation, as does nerve growth factor induction of NOS-1 in PC12 cells and Ca^{2+}-stimulated NOS-3 in endothelial cells. The MAPK signaling pathway in T-lymphocytes is activated by NO• donors via S-nitrosylation of the GDP–GTP exchange protein RAS on cysteine-118 with

subsequent recruitment and activation of the RAF-1 kinase (Deora *et al*., 2000). On the other hand, NO• inhibits the stress-activated kinase JNK by S-nitrosylation of cysteine-116. This appears to be the primary mechanism by which interferon-γ blocks JNK activation in macrophages (Park *et al*., 2000). Other proteins whose activities are modulated by S-nitrosylation include the P50 subunit of NF-κB, as discussed above (Marshall and Stamler, 2001), ryanodine, the Ca^{2+} release channel of sarcoplasmic reticulum (Eu *et al*., 2000), and mitochondrial caspases (Kim *et al*., 1997; Mannick *et al*., 1999; Mannick *et al*., 2001). An essential feature in activation of the FAS-dependent apoptosis pathway is the FAS-induced denitrosylation of caspase 3, freeing the active site cysteine (Mannick *et al*., 1999). The ROS scavenging properties and antiapoptotic functions of thioredoxin require S-nitrosylation of a specific cysteine (Haendeler *et al*., 2002).

The cysteine content of a protein is not the sole determining factor for S-nitrosylation (Hess *et al*., 2001). Specificity is seen in proteins with multiple cysteines, of which only one is S-nitrosylated by NO•. Analysis of S-nitrosylated proteins and the realization that acid–base catalysis alternatively promotes nitrosylation and denitrosylation led to a degenerate consensus sequence, (G,S,T,C,Y,N,Q,)(K,R,H,D,E,)C(D,E) (Stamler *et al*., 1997; Hess *et al*., 2001). This acid–base motif does not have to reside in the linear primary sequence, but, as has been shown for methionine adenosyl-transferase, can be generated from the tertiary structure of the protein (Perez-Mato *et al*., 1999).

This consensus sequence is found in hundreds of proteins beyond those mentioned above, including cyclin D1, Rb, BRCA1 and -2, and protein tyrosine phosphatases. Not all these proteins are S-nitrosylated and other factors that regulate nitrosylation include location of cysteines in hydrophobic compartments (Nedospasov *et al*., 2000; Hess *et al*., 2001). As noted above, a hydrophobic environment concentrates the lipophilic reactants, oxygen and NO•, thereby enhancing their rate of reaction and generating NO_x, including N_2O_3 that drives S-nitrosylation.

A potential role for S-nitrosylation in signal transduction pathway activation by ionizing radiation has been tested in some preliminary studies with the protein tyrosine phosphatases SHP-1 and SHP-2 (Figure 5, Barrett *et al*., in preparation). For this, Chinese hamster ovary cells were transfected with plasmids expressing either wild-type or mutants of these phosphatases in which the active site cysteine was mutated to a serine. After irradiation (4 Gy), S-nitrosylated proteins were purified according to the method of Jaffrey *et al*. (2001) and fractionated by gel electrophoresis. Western blots were probed with antibodies to either SHP-1 or SHP-2. The results in Figure 5 show a radiation-stimulated transient S-nitrosylation primarily of the active site cysteine of both phosphatases. Since previous investigations demonstrated NO• donor-stimulated S-nitrosylation of RAS, similar experiments were performed with cells transfected with a plasmid encoding RAS. Although basal S-nitrosylation of RAS was observed, no significant increase was observed after radiation.

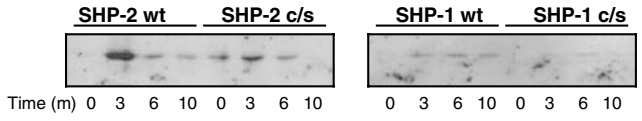

Figure 5 *S*-nitrosylation of protein tyrosine phosphatases. Chinese hamster ovary cells were transiently transfected with expression plasmids, encoding wild-type and dominant negative mutants of the protein tyrosine phosphatases SHP-1 and SHP2. At 48 h after transfection, cells were irradiated (4 Gy), and cell lysates were prepared at the given times postirradiation. *S*-nitrosylated proteins were purified according to Jaffrey *et al*. (2001) and fractionated by gel electrophoresis. Western blots were probed with appropriate antibodies

We estimate that approximately 5% of each phosphatase was S-nitrosylated after radiation. At least part of the low yield may reflect the inefficiency of the purification procedure. However, the catalytic activities of protein tyrosine phosphatases are in general 100-fold greater than protein tyrosine kinase activities (Tonks and Neel, 1996). Thus, only a very small change in phosphatase activity can translate into a substantial change in net tyrosine phosphorylation. Such transient S-nitrosylation and corresponding inhibition of protein tyrosine phosphatase may account for the enhanced levels of tyrosine-phosphorylated proteins after radiation and activation of signal transduction pathways that require protein tyrosine phosphorylation (e.g. Tuttle *et al*., 1998; Schmidt-Ullrich *et al*., 2000).

Of additional radiobiological interest are the mixed function protein tyrosine phosphatases that regulate the different cell cycle checkpoints. These phosphatases are also characterized by an active site cysteine. A recent study demonstrated that treatment of cells with relatively high concentrations of H_2O_2 ($>250\,\mu$M) stimulated degradation of CDC25C but not CDC25A. *In vitro* studies revealed that hydrogen peroxide stimulated disulfide bond formation between the active site cysteine-377 and an invariant cysteine at position 330 and suggested to the authors that oxidative stress independent of CHK1 activity may induce cell cycle arrest by inducing degradation of CDC25C (Savitsky and Finkel, 2002). In keeping with the homologies between protein tyrosine phosphatases, it would not be surprising to find that NO• *S*-nitrosylates and inhibits CDC25 family members, as found with SHP-1 and SHP-2. Such a mechanism may provide an additional layer of cell cycle regulation important in cellular responses to oxidative and nitrosative stresses and one that is initiated within the cytoplasm.

Conclusions: RNS as effectors of cellular oxidative events

Earlier in this review, we suggested that a two-step nitration mechanism for nitro-tyrosine formation also offers a hypothesis for cell signaling involving radicals. The first step entails a nonspecific oxidative reaction of highly reactive radicals of limited diffusion distance.

This in turn produces a more stable radical with chemical reactivity properties of high specificity consistent with a signaling molecule. In developing this hypothesis for biological signaling, we emphasized the importance of reactivity and relative concentrations of reactants. Highly reactive $OH^{\bullet}$ has a low target selectivity and diffusion distance. In contrast, $NO^{\bullet}$ and nitrite/nitrate are chemically inert with most biological molecules and exhibit a high degree of reaction specificity. We have also emphasized with several examples the importance of intracellular location for radical-driven cell signaling. Thus, the hydrophobic domains of membranes concentrate lipophilic reactants, such as $NO^{\bullet}$ and $NO_2^{\bullet}$, to generate the potent nitrosating agent, N_2O_3. Finally, the importance of RNS, such as $NO^{\bullet}$, in oxidative signaling can also be seen in the primary goals of cellular redox homeostasis mechanisms. Cells display an array of mechanisms for maintaining nonspecific highly reactive ROS at low manageable levels. On the other hand, cells precisely regulate RNS levels and thus RNS-stimulated signal transduction pathways through anabolic, catabolic and reversible storage mechanisms. For all the above reasons, $NO^{\bullet}$ can be considered the prototypic redox second messenger (Marshall *et al*., 2000; Hess *et al*., 2001).

Several studies have shown that cells in responding to a mild oxidative stress, for example, low concentrations of exogenous hydrogen peroxide or ionizing radiation, activate NOS activity. In analogy to the two-step mechanism for nitrotyrosine formation, we propose that cells convert a nonspecific oxidative event to a nitrosative signal. At present, the sensors for mild oxidative stress, including radiation-induced ionization events, have not been definitively defined. We suspect that mitochondria have a key sensing role because mitochondria generate most of a cell's ROS and thus one would expect mitochondria to be part of any homeostatic redox loop. There is also significant experimental evidence for mitochondria as oxidative sensors (e.g. Zorov *et al*., 2000; Leach *et al*., 2001). Conversion of the oxidative event to a nitrosative signal provides the amplification mechanisms that activate signal transduction pathways that are cytoprotective or, alternatively, in collusion with proapoptotic pathways induce cell death.

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