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Biological signaling by small inorganic molecules

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Abstract

Small redox active molecules such as reactive nitrogen and oxygen species and hydrogen sulfide have emerged as important biological mediators that are involved in various physiological and pathophysiological processes. Advancement in understanding of cellular mechanisms that tightly regulate both generation and reactivity of these molecules is central to improved management of various disease states including cancer and cardiovascular dysfunction. Imbalance in the production of redox active molecules can lead to damage of critical cellular components such as cell membranes, proteins and DNA and thus may trigger the onset of disease. These small inorganic molecules react independently as well as in a concerted manner to mediate physiological responses. This review provides a general overview of the redox biology of these key molecules, their diverse chemistry relevant to physiological processes and their interrelated nature in cellular signaling.

Graphical abstract

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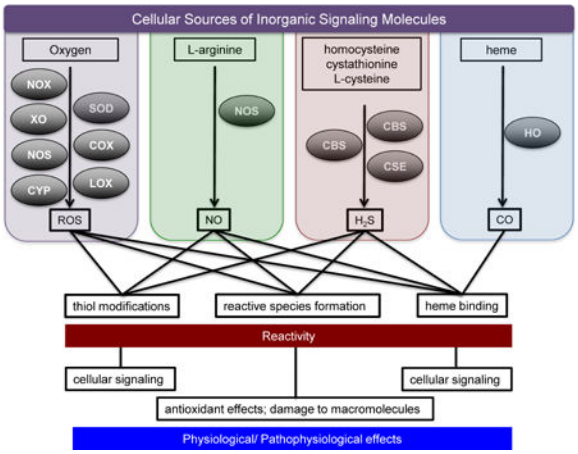
Dedication

This review is dedicated to Prof. Peter C. Ford whose contributions to inorganic chemistry include the areas of photochemistry, catalytic reactions and transition metal complexes. Prof. Ford's research interests span catalytic conversion of biomass feedstocks to chemicals and fuels, analysis of reactions of coordinated nitrogen oxides that are generated in mammalian biology and photochemical delivery of small bioregulatory molecules to physiological targets [121, 219, 383, 384]. His work on transition metal complexes with NO, NO_2^- and CO has provided model compounds for biological systems and has extended our understanding of the involvement of critical inorganic molecules in physiology and disease.



1. Introduction

Investigation of the biological effects of nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) initially concentrated on their context in environmental toxicology. For example NO and other nitrogen oxide species (often grouped as NO_x) are major components of air pollution [1], carcinogenic nitrosamines are found in food [2, 3] and H_2S and CO are industrial poisons [4, 5]. Given this history, the discovery of endogenous production of NO and related nitrogen oxides for eradication of pathogens including bacteria, parasites and viruses by the immune system and for regulation of physiological functions in the circulatory and nervous system was quite surprising [6–10]. The significance of the discovery of NO biosynthesis and its role in regulation of blood pressure was recognized by award of the 1998 Nobel Prize in Physiology or Medicine to Murad, Ignarro and Furchgott [11]. More recently, other small inorganic molecules, including CO, H_2S and even H_2, have also been identified as essential mediators of physiological processes. Their disequilibrium has been associated with disease onset and progression in cancer, immune response and cardiovascular function among others [12–14]. In addition, the diverse pharmacology of nitroxyl (HNO), for example in treatment of heart failure, alcoholism and cancer [15–19], strongly suggests that HNO is likely biosynthesized.

These signaling molecules are increasingly been referred to as endogenous gasotransmitters since they are all gases in pure form at STP. As gases, these molecules share important physical characteristics such as low molecular weight and neutral charge, which when coupled with lipophilicity, are important to their function as diffusible signaling agents. In the diverse environments of biological systems, the term gasotransmitter is a misnomer for these solute molecules. In addition, this term overlooks the fact that signaling is often indirect, for example after conversion of NO or H_2S into redox-related derivatives. Furthermore, the importance of O_2 is neglected because its production is not regulated, unlike NO, CO and H_2S.

Although redox activity is a major component of the signaling processes mediated by NO and H_2S, other reaction types, for example association of NO with heme systems, are also essential. Moreover, CO is not redox active. Here, we collectively consider the impact of

small inorganic molecules on signaling from the perspective of the direct and indirect effects of the neutral species: O_2, NO, HNO, CO, H_2S and H_2. A heavy emphasis is placed on the involvement of these and related species in redox signaling.

Redox active molecules play diverse and critical roles in all aspects of cell biology and physiology including metabolism, cellular signaling and host defense. Redox homeostasis is maintained by regulated production of redox active molecules, redox buffering and a diverse antioxidant system. Homeostatic regulation allows for initiation of signaling processes upon the interaction of reactive redox active species with specific targets. Under stress conditions, such as those often considered in chemical toxicology, overproduction of these species can induce damage to macromolecules including proteins, lipids and DNA. Antioxidants and other repair systems can promote survival under conditions of environmental stress; however, chemically induced stress can overwhelm such protective mechanisms creating an imbalance and leading to deterioration of cellular function. Since the boundary between normal and stress conditions is not precise, pathways exist to resolve the stressful incident and restore homeostasis or to induce cell death. Induction and control of stress-induced signaling by reactive redox active species is therefore important in both normal physiology and disease processes.

While the deleterious roles of small inorganic species, particularly reactive oxygen species (ROS), have been studied extensively, including impacts on pathological conditions such as aging and neurodegenerative diseases [20], analysis of their functions as signaling molecules is relatively new [21, 22]. Understanding of the fundamental chemistry of these species as well as the kinetic factors that control their generation and consumption is critical in order to build a realistic model for their participation in biological signaling mechanisms and physiological outcome.

Redox biology includes reactions of these chemical species with molecular targets, which have significant biological implications, especially in the context of cellular signaling. Often, cellular signaling pathways involve receptor-ligand interactions that rely on a structure-function relationship. In contrast, signaling by small inorganic messenger molecules typically involves covalent or coordinate covalent bond formation. Specificity occurs through spatial-, temporal-, and concentration-dependent constraints, typically via regulation of their biosynthesis pathway. The sources of these endogenous species are often metalloenzymes, and their targets can be considered to be primarily, although not exclusively, metal complexes and thiols [23–26]. While the reaction sites of these species overlap, their chemical and biological signatures are distinct, due to induction of different chemical modifications. In this review we discuss and highlight their biosynthesis, chemistry, and the interplay in and between subclasses of small inorganic signaling molecules.

2. Oxygen and Reactive Oxygen Species

Molecular oxygen serves as the ultimate electron acceptor during oxidative phosphorylation. Incomplete reduction of O_2 in the mitochondrial electron transport chain can lead to accumulation of ROS including superoxide (O_2^{-}), hydroxyl radical ($^{\cdot}OH$) and hydrogen

peroxide (H_2O_2), which as highly reactive species can be damaging to cellular macromolecules including lipids, proteins and DNA when produced in an unconstrained fashion [27, 28]. A variety of antioxidants including glutathione, flavonoids and vitamins A, C and E protect against ROS toxicity. Cellular levels of ROS are also tightly regulated enzymatically (Figure 1) [29–31]. Despite the known implications of ROS imbalance in the initiation of oxidative stress and disease, ROS regulate various biological and physiological processes. Understanding of the signaling cascades mediated by these species is therefore important [32].

One-electron reduction of O_2 is thermodynamically unfavorable (−0.33 V vs. NHE), which prevents indiscriminate oxidation. Direct reaction of O_2 with organic substrates is also kinetically inhibited due to ground state differences (i.e., spin restrictions). These kinetic barriers can be overcome by a variety of metalloenzymes, such that activation of O_2 is coupled to diverse metabolic transformations. Hydroxylation reactions, for example during purine catabolism by xanthine oxidase, are well known to lead to O_2^- production [33]. Rather than simply being a byproduct, O_2^- derived from xanthine oxidase can be utilized to oxidize other species such as cytochrome *c* [34]. Both xanthine oxidase and cytochrome *c* are commonly used experimentally to produce and detect O_2^-, respectively.

The toxicity of O_2^- is harnessed by the immune system to eradicate pathogens. NADPH oxidase is a transmembrane multienzyme complex that couples oxidation of NADPH with reduction of O_2 in neutrophil phagosomes. Identification of distinct isoforms in a variety of cell types, collectively referred to as the NOX family [29, 35–37], have suggested a role for O_2^- production beyond host defense. Regulated production of ROS by NOX proteins has now been implicated in cell signaling processes including post-translational modifications and regulation of gene expression [38].

Oxidation or the absence of tetrahydrobiopterin, a cofactor for nitric oxide synthase (NOS), leads to uncoupling of the dimer and production of O_2^- [39–41]. Formation of O_2^- by the reductase domain of NOS is implicated in pathological conditions for example stemming from endothelial dysfunction [42–45]. More recently, a tight correlation has been demonstrated between NO biosynthesis and tetrahydrobiopterin in a number of different endothelial and vascular smooth muscle cell lines. However, the molecular mechanism of this effect is still unknown [46–48]. Uncoupling of the catalytic turnover of P450 enzymes can also lead to production of O_2^- by autoxidation of the ferrous dioxygen complex. Formation of ROS from P450s has been previously reviewed [49, 50].

The metabolism of arachidonic acid and polyunsaturated fatty acids by cyclooxygenases (COX) and lipoxygenases (LOX) can also generate ROS and radical species in their pathways. LOX-dependent production of O_2^-, initially suggested by Lynch and Thompson [51], has been demonstrated in skeletal muscle and endothelial cells [52–54]. Generation of O_2^- by COX has been suggested to impair K^+ channels after brain injury and G protein-mediated cerebrovasodilation [55, 56]. While the mechanism of O_2^- formation is unknown, it is hypothesized to form by electron transfer from fatty acid hydroperoxides to O_2 [57]. This reaction can be favorable if the fatty acid radical formed in the process is stabilized by resonance delocalization. The involvement of unsaturated fatty acids in production of O_2^- in

macrophages has also been suggested [58]. In 1986 Kukreja *et al*. showed that NADPH or NADH is required for O_2^- formation by COX and LOX [59].

Three important factors that dictate whether ROS formation will lead to redox signaling or oxidative damage are the site of formation, the reaction rate with target species and the rate of detoxification. With respect to reactivity, O_2^- is a mild reductant, a strong oxidant and a free radical. The reduction of Fe^{3+} to Fe^{2+} ion by O_2^- was described by Haber and Weiss in the 1930s [60] (Eq. 1).

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad 1)$$

O_2^- can also react with a variety of both endogenous and exogenous reducing agents (e.g., thiols, ascorbate, semiquinones [61–63]) as well as other free radicals. The latter reaction is typically far more facile than the conversion of reducing agents into free radicals (e.g., near diffusion controlled reaction of O_2^- with NO vs. oxidation of thiols to the thiyl radical at ~10^3 M^{-1} s^{-1} at pH 7.4 [64]). However, the overall rate of these reactions will dependent on the concentration of the reactants. The reaction of NO for instance would only be kinetically viable in close proximity to cells that are actively producing NO while thiols are relatively stable and abundant. Either reaction type may be considered to be protective in that free radicals are consumed. However, the product of the reaction of O_2^- with NO is itself a reactive species (see section 3 for further details) and oxidation of redox active biomolecules can propagate free radical formation [65].

Since one-electron reduction of O_2 is thermodynamically unfavorable while one-electron reduction of O_2^- is quite favorable (0.89 V vs. NHE), O_2^- is unstable to disproportionation producing O_2 and H_2O_2 (5×10^5 M^{-1} s^{-1} at pH 7.0). Superoxide dismutases (SODs) significantly accelerate disproportionation [66], maintaining low physiological concentrations of O_2^-. Again, it is important to note that H_2O_2 is also a reactive species. H_2O_2 may be formed by other pathways, for instance through two-electron reduction of O_2 by xanthine oxidase [67], NADPH oxidase [68] and sulfhydryl oxidase, which also converts thiols to disulfides [69], and by one-electron reduction of O_2^- [61, 70].

Similarly to O_2^-, H_2O_2 can react with metals and cellular reductants and can be consumed enzymatically. The interaction of H_2O_2 with iron complexes in particular is important. Fenton's reagent, or hydrogen peroxide and Fe^{2+} (Eq. 2), was developed in the late 1800s for hydroxylation of organic compounds and oxidation of organic contaminants [71].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad 2)$$

Reduction of Fe^{3+} to Fe^{2+} ion by O_2^- (Eq. 1) then provides a catalytic mechanism to produce powerful oxidants such as $^{\cdot}OH$ via Eq. 2 [70, 72]. Other metal ions such as Cu^+, Ti^{3+}, Cr^{2+}, Co^{2+} also undergo similar reactions with H_2O_2 and are known as Fenton-like reagents [73].

The importance of this reaction was expanded with the discovery that oxidants derived from Fenton chemistry could alter macromolecules with pathological consequences. Fenton-like reactions are now considered to be primary causes of oxidative stress [74]. Since a rich literature was concurrently accumulating with respect to generation of oxidants and reactive molecules from ionizing radiation, it was commonly assumed that ˙OH was associated with both ionizing radiation and the Fenton-like reaction. However, in the 1950s Henry Taube suggested that the oxidant was instead a hypervalent metal-oxo species, as had been earlier described by Bray and Gorin (Eq. 3) [75, 76].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{IV}{=}O + H_2O \quad 3)$$

The nature of the oxidant has since been controversial [77–79]. Formation of Fe^{3+} is rate-limiting, suggesting that Eq. 2 represents a concerted reaction [80]. However, in the early 1990s, stopped flow analysis led to detection of an intermediate species prior to formation of Fe^{3+} during oxidation of N-dimethyl nitrosamine [80–82]. This intermediate was determined to be an aqueous iron nitrosyl complex, indicating that an oxidant is produced as an intermediate prior to formation of Fe^{3+} [82]. These results supported prior suggestions of the metal-based oxidants during the classical Fenton reaction [75, 76]. Competition experiments also indicated that iron peroxo complexes led to similar chemical modifications as to ˙OH, as proposed by Drago and colleagues with cobalt complexes [83]. The ligand environment may influence the identity of the major oxidant, with hard ligands perhaps promoting ˙OH, while heme favors hypervalent species [75, 76, 80].

As with O_2^-, H_2O_2 can oxidize thiols, but the product is a sulfenic acid (Eq. 4) [84]. The reaction rate is highly dependent on the protein microenvironment (10–10^6 M^{-1} s^{-1}), which affects thiol nucleophilicity.

$$RS^- + H_2O_2 \rightarrow RSOH + OH^- \quad 4)$$

Reductants such as ascorbate can reverse this modification through an addition/elimination mechanism [85]. Furthermore, sulfenic acids can undergo further oxidation to sulfinic acid (Eq. 5), which in turn are susceptible to oxidation to sulfonic acids (Eq. 6) [86].

$$2RSOH \rightarrow RSO_2H + RSH \quad 5)$$

$$RSO_2H + H_2O_2 \rightarrow RSO_3H + H_2O \quad 6)$$

Sulfenic acids can also react with excess thiols to form disulfide bonds or with proximal amines to form sulfonamides ($RSNR_2$). The diversity of thiol modifications is extensive, and investigation of the implications of such modifications on cellular signaling is ongoing. For a review of the ROS-mediated alterations of critical cysteine residues, see Klomsiri *et al*. [87].

Chance demonstrated in 1949 that H_2O_2 was consumed in the presence of catalase [88–91] in a disproportionation reaction to water and O_2. Other antioxidant enzymes, which reduce H_2O_2 to H_2O include peroxiredoxins and glutathione peroxidases [92–96]. Although H_2O_2 is less reactive than O_2^-, control of H_2O_2 levels is critical given that H_2O_2 can readily diffuse across cellular membranes, and thus can have an extended sphere of influence. Active transport is also possible through members of the aquaporin family [97, 98].

Although production of H_2O_2 is well known to lead to oxidative damage of macromolecules and to be an underlying cause of numerous diseases and aging, a role for H_2O_2 as a messenger molecule in redox signaling is emerging. For instance post-translational modifications of cysteines lead to allosteric changes that alter protein function [31, 99]. The pathophysiological implications of H_2O_2 signaling in cancer, inflammation and aging have been extensively reviewed by Schieber *et al*. [100]. Here, we note that such pathways place metalloproteins like NADPH oxidase, which produces H_2O_2, and catalase, which consumes H_2O_2, in a highly regulated manner in a central role [101, 102].

As a highly reactive molecule, $^{\cdot}OH$ can react with a diverse variety of molecules including lipids, proteins, DNA, RNA and carbohydrates with a near diffusion controlled rate constants without specificity, thus contributing to widespread deleterious effects. However, $^{\cdot}OH$ may also be involved in cellular signaling. Reaction with lipids such as arachidonic acid leads to formation of isoprostanes [103, 104] and α,*β*-unsaturated aldehydes [105]. Isoprostanes have been implicated in various disorders and pathophysiological conditions [106]. Acrolein, 4-hydroxy-2-nonenal and crotonaldehyde are α,β-unsaturated aldehydes that are highly toxic and promote oxidative stress-mediated signaling associated with pathophysiological conditions by reacting with free thiol and amine groups of other macromolecules [107–109]. These reactions, along with the reactions of $^{\cdot}OH$ with proteins and DNA are well summarized by Marnett *et al*. [110].

3. The Chemical Biology of NO

While ROS primarily arise from reduction of O_2, reactive nitrogen species (RNS) can be generated by oxidation of NO, which itself is produced by oxidation of arginine by NO synthases (NOS) (Figure 2) [111].

Three isoforms have been identified: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3). The constitutive isoforms eNOS and nNOS produce low cellular levels of NO, generally in response to transient changes in calcium levels, while induction of iNOS can lead to sustained NO fluxes in the micromolar range. Under hypoxia, reduction of nitrite (NO_2^-) can provide an alternative, oxygen-independent source of low levels of NO (for example, [112]).

Intense interest in the chemistry of NO and related RNS emerged following the identification of NO as an important mediator of both physiological and pathophysiological processes [7–10]. Unlike many signaling agents, which rely on receptors where structural relationships determine function, the chemistry of RNS determines biological activity. Since the lifetimes of RNS are limited, the kinetics of these reactions determine biological outcomes. Defining these reactions within the context of physiology and disease is

challenging. A simplifying concept developed in the 1990s is *the chemical biology of NO* [113–115], which identifies two distinct reactions types based on NO concentration, reactive species and reaction kinetics (Figure 3).

3a. Direct effects of NO

Direct effects involve interaction of NO itself with biological targets. These reactions are facile with rate constants $>10^5$ M^{-1} s^{-1} and primarily involve heme proteins and other radical species. Perhaps the most important example of a direct effect is coordination of NO to the heme protein soluble guanylyl cyclase (sGC). This association leads to the majority of the physiological effects of NO through formation of cGMP from GTP [116]. Nitrosylation of ferrous sGC (7×10^8 M^{-1} s^{-1}) [117] labilizes the axial His^{105} ligand [118] due to the strong trans effect of NO. The resulting structural change leads to significant enhancement of activity [119, 120].

While loss of NO from ferrous heme complexes tends to be very slow [121], release of NO from sGC is critical for reversible activation. The slow rate of NO dissociation measured with purified sGC has been puzzling [122]. Recent studies indicate a dependence on ATP and GTP [123] suggesting that sGC deactivation may be complex [122, 124, 125].

NO can interact with a number of other heme proteins including hemoglobin, cyclooxygenase, cytochrome P450 and cytochrome *c* oxidase [126, 127]. NO can either bind directly to the iron center or can interact with other bound ligands. For instance, the reaction of NO with oxyhemoglobin leads to formation of nitrate (NO_3^-) and methemoglobin (3.4×10^7 $M^{-1}s^{-1}$) [128] through interaction with ligated O_2. This reaction is generally considered to serve as a major regulatory mechanism of NO flux [129]. NO can also react directly with hypervalent species formed during the Fenton reaction (Eq. 3) to reduce the metal to a normal oxidation state (Eq. 7) [130, 131].

$$Fe^{IV}{=}O + NO \rightarrow Fe^{3+} + NO_2^- \quad 7)$$

The concentration and duration of NO is important with respect to interactions with metal centers. In addition to kinetic factors, the thermodynamic stability of the metal-nitrosyl bond is important in determining the extent of signaling. NO has a higher affinity for Fe^{2+} compared to Fe^{3+}. Several summaries of rate constants for Fe^{2+} and Fe^{3+} heme interactions have been reported [126, 127, 132, 133]. A quick rule of thumb for the flux of NO required for activation is to take the reciprocal of the K_{eq}, which is k_{on}/k_{off}. This provides a ballpark estimate for the importance of a reaction under defined conditions. For example, K_{eq} ~10^9 M^{-1} for sGC indicates that a flux of 1–10 nM is required for pathway activation. In contrast, Fe^{3+} proteins such as COX2 have K_{eq} ~10^2 M^{-1}, indicating that the formation of a stable nitrosyl complex requires a 100 μM steady state NO flux, which is not readily achieved *in vivo* [127].

As a free radical, the reaction of NO with other free radicals is typically facile. An important exception is the dimerization of NO, which is only favored at low temperatures of high pressures. Association of NO with other free radicals in biological systems often serves an

antioxidant role abating propagation cycles and associated toxicities [113, 114]. Reaction of NO with O_2^- and NO_2 results in alteration of the ROS/RNS profile and thus is described in more detail in the following section.

3b. Indirect effects of NO

The concentration of NO produced by NOS dictates the reactive species that are produced as well as their downstream targets. Interaction of NO at nanomolar levels with sGC promotes vasorelaxation [9, 134, 135]. In contrast, higher concentrations of NO can lead to interactions with other metal centers or to formation of RNS typically by oxidation of NO. These species mediated the indirect effects of NO by modifying unique biological targets. Such reactions are critical in anti-pathogen and anti-tumor responses of the immune system [6, 136–140]. The major RNS include nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4) and $ONOO^-$. These reactive species are potent nitrating, nitrosating and oxidizing agents [113, 141] that play important roles in redox biology and sGC-independent signaling.

RNS formation pathways include autoxidation of NO (Eq. 8)

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad 8)$$

The mechanism of NO autoxidation is still a matter of debate, but both NO_2 and N_2O_3 are often invoked (Eqs. 9, 10).

$$2NO + O_2 \rightarrow 2NO_2 \quad 9)$$

$$NO + NO_2 \rightleftharpoons N_2O_3 \quad k_f = 1.1 \times 10^9\,M^{-1}\,s^{-1}, k_r = 8.1 \times 10^4\,s^{-1} \quad 10)$$

Autoxidation of NO has a reasonably high rate constant ($4.8 \times 10^6\ M^{-2}\ s^{-1}$ [142]) but is second order in NO. This dependence may limit the significance of the reaction to conditions of high NO such as inflammation. Furthermore, that both NO and O_2 partition to nonaqueous environments suggests that NO autoxidation may be confined to hydrophobic lipid membranes.

Once formed, N_2O_3 can undergo rapid hydrolysis to form nitrous acid (HNO_2) (Eq. 11) [143], which is a weak acid (pK_a of 3.4).

$$N_2O_3 + H_2O \rightleftharpoons 2HNO_2 \quad 11)$$

The reversibility of Eqs. 10 and 11 leads to a mechanism for conversion of nitrite to NO, for example in acidic environments such as the stomach [144–146], and demonstrates the interrelated nature of NO and RNS.

Formation of NO_2 and N_2O_3 can lead to oxidation/nitration/nitrosation of biomolecules such as proteins and lipids. The principal mediator of RNS-associated oxidative stress is NO_2.

The toxic effects of NO_2 are well known for example in air pollution and cigarette smoke [1]. It is associated with irritation of the nose and eye, as well as pulmonary edema and bronchiolitis. Nonetheless, NO_2 is also a key component of the immune system during pathogen eradication [147, 148]. NO_2 induces nitration of tyrosine, and this post-translational modification is associated with inflammation and disease processes [149, 150]. Formation of nitrotyrosine is a central feature of NO_2 chemistry and is used as a biomarker for NO_2 [151, 152]. Several studies suggest that nitration occurs on specific residues rather than indiscriminately [153, 154]. While tyrosine nitration is a well-established phenomenon, the underlying routes of NO_2 formation and the impacts of tyrosine nitration on protein function are less clear. Readers are referred to excellent reviews on the link between protein nitration and disease etiology [155, 156].

The primary mediator of nitrosative stress is N_2O_3, which promotes a variety of non-radical reactions with thiols and amines. Nitrosation of secondary amines can produce carcinogenic nitrosamines [7]. Nitrosation of proline has been observed in gastric cancer patients and may provide strong evidence of N_2O_3 generation *in vivo* under conditions of inflammation [157, 158]. Nitrosation of thiols can inhibit enzymatic function and can activate key signaling pathways under normal and pathophysiological conditions [159, 160]. For example, N_2O_3 inhibits DNA repair proteins by modification of key thiol residues, degradation of zinc finger proteins, and nitrosation of lysine residues for example in ligases, which results in deamination [161–166]. In contrast, nitrosation of growth factors and membrane thiols can activate important pro-growth signaling mechanisms that promote tissue restoration as well as inflammatory disease processes [167]. For review of the implications of nitrosation in physiological and pathophysiological processes see Anand *et al.* [168].

Formation of an *S*-nitrosothiol is often used as a nitrosative footprint [169], but pathways other than interaction of thiols with N_2O_3 can lead to *S*-nitrosothiol production [170]. A large body of literature suggests *S*-nitrosothiols as a principle mediator of sGC independent cellular signaling. A detailed discussion on RSNO is beyond the scope of this review, and readers are referred to several other reviews [171–173].

Similarly to NO autoxidation, the rapid reaction of NO and O_2^- (Eq. 12; 6.7×10^9 M^{-1} s^{-1} [174] is a prime example of the interrelated nature of RNS and ROS.

$$NO + O_2^- \rightarrow ONOO^- \quad 12)$$

In addition to modulation of the bioavailability of both NO and O_2^- (thus preventing formation of H_2O_2), the product $ONOO^-$ is highly reactive [175–177] and can be converted to other RNS. Although $ONOO^-$ can undergo isomerization to form NO_3^- ($1\ s^{-1}$) in a deactivation pathway, homolytic cleavage to form NO_2 and $^{\cdot}OH$ is significant (30% yield) [178]. Also, $ONOO^-$ reacts with CO_2 leading to formation of $ONOOCO_2^-$ (Eq. 13; 2.3×10^3 M^{-1} s^{-1}) [179], which can further decompose to NO_2 and the carbonate radical (Eq. 14), which are powerful oxidants.

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad 13)$$

$$ONOOCO_2^- \rightarrow [NO_2|OCO_2^-] \rightarrow NO_2 + CO_3^- \quad 14)$$

In the presence of excess NO, Eq. 8 can again be invoked, leading to formation of N_2O_3. Such reactions impact the balance between ROS and RNS and provide a spectrum of oxidative and nitrosative chemistry. The chemistry will be modulated by the relative concentrations of NO and O_2^- and consumption pathways. For example, high SOD activity competes with reaction of NO.

Several studies have shown an antagonistic relationship either chemically or through regulation of enzymatic sources of ROS or NO. In the case of NADPH oxidase, a major source of ROS, NO inhibits assembly in an isoform-specific manner, leading to reduced ROS levels [180–182]. Furthermore, the interactions of NO and ROS can modulate signaling pathways. For example, both NO and ROS stabilize the stress protein p53, but NO moderates ROS–mediated p53 stabilization and similarly ROS abates NO p53 stabilization [183, 184]. Taken together this redox balance provides an important component to overall cellular and physiological signaling and toxicity. As such, the antioxidant effects of NO have been exploited in preventing and treating stroke and myocardial infarctions [185–187].

3c. Role of NO in breast cancer

NO has a concentration-dependent role in cancer biology [188]. Intermediate NO concentrations play a critical role in regulation of tumor growth, migration and metastasis [189, 190]. NO levels between 200–700 nM flux for several hours lead to cellular proliferation and properties associated with metastasis of estrogen receptor negative (ER(−)) breast cancer cells [189]. In contrast, such NO fluxes regulate wound-healing responses critical for tissue restoration in normal cells [191]. Moreover, in cancer an NO flux of ~300 nM promotes activation of the extracellular signal-regulated protein kinase (ERK) and phosphoinositide 3-kinase (PI3k)/protein kinase B (Akt) pathways, as well as stabilization of hypoxia-inducible factor-1 (HIF-1α) [192–194]. Two intriguing mechanisms lead to these cellular responses. One involves nitrosation of specific thiol residues of membrane bound proteins including SRC, EGFR, and ERK by autoxidation products of NO [193], while the other involves nitration of tyrosine residues. Interestingly, TIMP1 an endogenous inhibitor of matrix metalloproteinases (MMPs) can be nitrated at key tyrosine residues that prevent its inhibitory function of active MMPs [195] while promoting activation of PI3k/Akt pro-survival signaling [196].

3d. Delivery of exogenous NO

There are several methods for delivery of NO [197, 198]. NO-based anti-cancer therapeutic applications require >800 nM steady state NO levels for prolonged periods of time [199]. Such flux levels systematically lead to unwanted cardiovascular side effects including a hypotensive response and impaired platelet function since proper cardiovascular function requires a significantly lower flux of NO. Therefore, therapeutic levels of NO must be delivered site-specifically for oncologic applications. Several strategies exist, including

development of prodrugs. One of the most reliable and versatile classes of NO donor is diazeniumdiolates, which are often referred to as NONOates [200]. These compounds spontaneously decompose in a controlled, time-dependent fashion to yield specific fluxes of NO. Prodrug technology can significantly increase the half-life of NONOates, as well as facilitate site-specific delivery. For example, liver specific enzymes activate V-PYRRO/NO while JS-K is activated by glutathione transferase and shows significant anti-cancer properties in various cell lines [201]. Recently, NO-releasing nanoparticles have shown promise due to their ability to release high payloads of NO [202–204].

Another attractive delivery strategy involves photochemical release of NO from metal nitrosyl complexes [205, 206]. A major challenge of this strategy is the design of complexes with high intensity absorption bands at low energy, since longer wavelengths of light penetrate skin to greater depths than those of higher energies. Toward this end, Ford and coworkers have shown that Fe-S complexes such as Roussin salts can radiosensitize hypoxic cells upon photochemical release of NO [207]. More recently, quantum dots have been adopted to release NO [208].

3e. NO and nitrite

Nitrite can be reduced to NO under hypoxic conditions, leading to dilation of blood vessels and increased blood flow [209, 210]. Study of the reaction of nitrite with heme proteins dates back several decades [211]. Nitrite stored in tissue is reduced by Fe^{2+} complexes to produce NO [212–214]. This provides an important protective mechanism against tissue ischemia because the O_2-dependence of NOS activity dictates reduced levels of NO under hypoxic conditions [112, 215, 216].

One of the most important aspects for cardiovascular health is the role of oral and gastrointestinal microbiota, which provides an important nitrite/NO reduction cycle [217, 218]. Nitrate from food such as spinach or beet juice is converted to nitrite in the mouth. Upon swallowing, in the acidic environment of the stomach, disproportion of nitrous acid leads to NO, which has beneficial effects such as reducing gut ulceration. Nitrate in the circulating blood is secreted again through the saliva glands and can be reduced to nitrite. Maintenance of circulating and tissue nitrite provides a buffer against hypoxia, which can promote vasodilation and decrease thrombosis [218]. Thus, metal-mediated reduction of nitrite is important in maintaining cardiovascular health and in part explains why a vegetable rich diet is a positive health factor for prevention of cardiovascular disease and cancer.

Ford and colleagues examined the reaction of nitrite with a variety of metal and porphyrin-like complexes and have identified a novel mechanism involving the transfer of oxygen to form NO [219]. Moreover, this intriguing mechanism may produce nitroxyl (HNO) as well [220]. This may provide a unique mechanism to regulate the NO/HNO balance, which mediates different physiological outcomes.

4. The Chemical Biology of HNO

HNO is a nitrogen oxide that has been studied since the late 1800s [221–223]. In addition to extensive studies on structural and spectroscopic parameters, the number of reports on the

pharmacological effects HNO has been expanding since the turn of this century. Discovery of unique biological properties have led to a resurgence of interest, particularly in understanding the chemical biology of HNO and comparison of physiological effects with the redox sibling NO [18, 224].

Although several studies postulate mammalian biosynthesis of HNO, this remains highly debated, in part due to inefficient/nonspecific *in vivo* detection systems. Possible routes for endogenous production of HNO include but are not limited to oxidative degradation of the NO biosynthesis intermediate N^{ω}-hydroxy-L-arginine (NOHA), release from NOS in the absence of the cofactor tetrahydrobiopterin, reaction of *S*-nitrosothiols with thiols, reduction of NO by metalloproteins such as MnSOD, xanthine oxidase or cytochrome *c*, from the reaction of H_2S with HSNO (thionitrous acid, the smallest *S*-nitrosothiol), and most recently from nitrite via heme-iron-catalyzed metabolism of H_2S [225–234]. Regardless of the open question of biosynthesis, numerous studies have shown the potential of HNO to be used in pharmacological settings such as cardiovascular disorders, inflammation, alcoholism, cancer and pain [15–17, 235–238].

The chemistry of HNO has several interesting aspects. First, HNO is metastable and undergoes rapid dehydrative dimerization (Eq. 15; $8 \times 10^6\ M^{-1}\ s^{-1}$) [239–241].

$$2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O \quad (15)$$

This self-consumption pathway both precludes storage and limits the reactivity and lifetime of HNO. Furthermore, the production of N_2O is also often considered as an indirect marker of HNO. Although HNO is a weak acid (pK_a of HNO > 11 [242–244]), the rate of proton transfer between the acid-base pair is severely constrained by a spin difference (^{1}HNO and $^3NO^-$). The spin forbidden relationship coupled with a self-consumption pathway not only inhibits establishment of an acid-base equilibrium but also dictates that resulting chemistry is likely from the initial species (HNO or NO^-) rather than from a combination of the two. For a detailed discussion, see [245].

The pK_a of HNO is actually derived from the reduction potential for the NO/NO^- couple of −0.8 V [242, 243]. At physiological pH, the reduction potential will be increased to −0.5 V. Such a potential is nearly inaccessible biologically, indicating that NO reduction is unlikely. In contrast, oxidation of HNO to NO is thermodynamically favorable [246, 247]. However, the proton-coupled process is kinetically constrained, such that other reactions are more likely, particularly with thiols and higher valent metals [245, 248]. A number of studies have now firmly established unique and distinct chemical biology of HNO compared to NO [18, 127, 245, 249]. Nonetheless, the potential for conversion of HNO to NO under biological conditions [250–252] should be considered when assessing the effects of exposure to HNO.

4a. HNO and thiols

As an electrophile, HNO can potentially react with a variety of biological nucleophiles. Unlike NO, thiols represent a major site of direct reactivity for HNO under physiological conditions [18, 229]. Association of HNO with thiols generates *N*-hydroxysulfenamide (Eq.

16). In the presence of excess thiol, this unstable intermediate can further react with a second thiol (Eq. 17) to produce disulfide and NH_2OH. On the other hand, the *N*-hydroxysulfenamide can rearrange to produce a sulfinamide (Eq. 18) [229, 253]. The disulfide product represents a biologically reversible process as biological reductants regenerate the thiol while generation of sulfinamide is unique in terms of HNO chemical biology and represents an irreversible modification of thiols [249, 254].

$$\mathrm{RSH+HNO \rightarrow RSNHOH} \quad 16)$$

$$\mathrm{RSH+RSNHOH \rightarrow RSSR+NH_2OH} \quad 17)$$

$$\mathrm{RSNHOH \rightarrow RS(O)NH_2} \quad 18)$$

Many of the physiological actions of HNO can be explained on the basis of modification of critical thiols. For instance, inhibition of enzymes such as aldehyde dehydrogenase (ALDH), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the zinc finger protein poly(ADP-ribose) polymerase (PARP) can explain HNO effects respectively on alcohol metabolism, glycolysis and apoptosis [161, 255, 256].

4b. HNO and metal proteins

The relative stability of ferrous compared to ferric nitrosyl complexes is well recognized [121, 126]. Unlike NO, HNO does not readily form stable complexes, although Fe(II)HNO heme protein complexes have been isolated [257, 258]. Compared to NO, the association of HNO is generally more favorable with Fe^{3+} rather than Fe^{2+} [18, 259, 260]. The reaction of HNO with Fe^{3+} hemoproteins leads to formation of the corresponding ferrous nitrosyl species via reductive nitrosylation (Eq. 19) [261, 262].

$$\mathrm{HNO+Fe^{3+} \rightarrow Fe(II)NO+H^+} \quad 19)$$

The concomitant spectral shifts upon reductive nitrosylation of metmyoglobin are often used to confirm HNO production from donor compounds [260]. Other than globins, cytochromes and peroxidases also undergo reductive nitrosylation by HNO [18, 263] as do other metal complexes [264]. Reductive nitrosylation can also lead to formation of free NO depending on protein identity. For instance axial occupation in ferricytochrome *c* or d^{10} metal centers such as in Cu,Zn SOD reduces nitrosyl complex stability [250, 262].

Contrary to their different reactivity toward Fe^{3+} species, both NO ($4 \times 10^7\ M^{-1}\ s^{-1}$) and HNO ($1 \times 10^7\ M^{-1}\ s^{-1}$) react facilely with Fe(II)O_2 complexes such as oxymyoglobin to give the ferric protein [18, 265, 266]. The mechanism of this reaction for HNO has not been fully established, due in part to the lack of complete end product characterization. Regardless of the mechanism, NO and HNO can be differentiated by using a scavenger such as the thiol glutathione (GSH), which quenches HNO chemistry [18].

A primary and well established biological target for NO is the Fe^{2+} heme protein sGC [267]. Based on the observation that HNO donors induce vasorelaxation, speculation has been offered as to whether HNO can interact with sGC in a manner similar to NO [225, 268]. HNO has recently been implicated as an activator of sGC by interacting with the ferrous heme and with thiol residues [269]. Unlike NO, the trans effect of HNO is not strong enough to cleave the distal Fe-His bond, thus implying that sGC mediated cellular signaling is tightly controlled [270]. The sGC-dependent vasodilatation and sGC-independent positive inotropy of HNO has long been studied as a viable option for treatment of cardiovascular disorders [271–273]. Recently, HNO mediated activation of sGC showed suppression of cardiomyocyte hypertrophy and O_2^- generation, thus further emphasizing its role in cardiovascular pharmacotherapy [274].

4c. HNO and O_2

Overwhelming data suggests that HNO and O_2 react to form an oxidant that differs from synthetic $ONOO^-$ [275–278]. Unlike the reaction of NO and O_2^- or bolus peroxynitrite, free radicals are not produced in the reaction of HNO with O_2 as verified by the absence of protein nitration or dimerization of phenolic compounds [275]. However, oxidation of ferrocytochrome *c* by HNO indicates that the product of HNO autooxidation may induce one-electron oxidation under certain circumstances [279]. Observation of hydroxylation suggests the presence of a strong two-electron oxidant. In addition, HNO autoxidation can induce DNA double strand breaks while $ONOO^-$ does not induce similar damage [276, 280–282]. Despite these differences, use of a boronate probe has led to the proposal that the oxidation product of HNO and O_2 is in fact $ONOO^-$ [283, 284]. One possible explanation is that different protonated isomers may be formed. A recent calculation suggests $HN(O)(O_2)$ as an intermediate of HNO autoxidation [285]. This species would be expected to be an oxygen atom transfer agent culminating in two electron-oxidation rather than radical chemistry. The challenge to this potential hypothesis is how fast the nitrogen deprotonates to give $ONOO^-$. This reaction warrants further examination.

4d. HNO and NO

HNO and NO react with each other to form reactive intermediates (Eq. 20–22). However, these reactions are biologically feasible only in the presence of low concentration of scavengers such as GSH since the rate constants for reaction of HNO with GSH (2×10^6 M^{-1} s^{-1}) [18] or for dimerization (Eq. 15; 8×10^6 M^{-1} s^{-1}) are similar to that of reactions 20–22.

$$HNO + NO \rightarrow HN_2O_2 \quad 6 \times 10^6\,M^{-1}\,s^{-1} \quad 20)$$

$$HN_2O_2 + NO \rightarrow HN_3O_3 \quad 8 \times 10^6\,M^{-1}\,s^{-1} \quad 21)$$

$$HN_3O_3 \rightarrow N_2O + HNO_2 \quad 2 \times 10^4\,M^{-1}\,s^{-1} \quad 22)$$

The discussions above indicate that the biological chemistry of HNO is rich and diverse. Effects associated with HNO must be analyzed in light of direct interaction with primary targets such as thiols and metalloproteins or indirect effects that arises from its interaction with NO and O_2.

4e. Delivery of exogenous HNO

As with NO, donor compounds are extensively used to produce HNO [286, 287]. Commonly used donors include Angeli's salt ($Na_2N_2O_3$) [241, 288], Piloty's acid ($C_6H_5SO_2NHOH$) and derivatives [289, 290], acyl nitroso compounds [291–293], acyloxy nitroso compounds [294–296] and primary amine based diazeniumdiolates [RNH-N(O)=NO^-] [297, 298]. Among these donor classes, Angeli's salt is the most prevalent donor used for chemical and biological studies. Angeli's salt has been well characterized and is a spontaneous donor of HNO [241]. Piloty's acid and derivatives are useful base-sensitive HNO donors whose HNO release profile can be tuned by varying the organic substituent. A tendency to generate NO instead of HNO under aerobic conditions has limited usage of Piloty's acid and derivatives [289]. More recently, HNO donors based on acyloxy nitroso compounds and diazeniumdiolates have been developed [299–301]. Recent examination of the acetoxymethyl and aspirin derivatives of isopropylamine diazeniumdiolate (IPA/NO) demonstrated that HNO could be site-specifically delivered to cancer cells [237, 301]. The clinical potential of HNO-based therapeutics warrants development of new HNO donors.

5. The Chemical Biology of H_2S

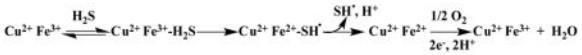
H_2S is mainly formed by anaerobic digestion of organic matter. The toxic and environmental effects of H_2S have been extensively studied over the last century. Endogenous production of H_2S in combination with its physiological and pathophysiological roles in mammalian systems has led to renewed interest in understanding its biological function [25]. H_2S is endogenously produced in mammalian tissue mainly by cystathionine β-synthase (CBS) [302, 303] and cystathionine γ-lyase (CSE) [304, 305] from L-cysteine, homocysteine, and cystathione (Figure 4). H_2S is also produced from 3-mercaptopyruvate by the action of 3-mercaptopyruvate-S-transferase (MST) [306].

As a physiological mediator [307], H_2S is involved in calcium homeostasis, neuromodulation, inflammatory response, cardiovascular and gastrointestinal function [308–314]. The physiological concentration of H_2S is estimated to be on the order of 15 nM [315], and its levels are tightly controlled. With a pK_a of 6.8, both H_2S and HS^- exist under physiological conditions. As it is not currently known whether H_2S, HS^- or both are biologically active, it is common to simply refer to H_2S as a collective term. Like NO and HNO, H_2S also has various biological targets, and thus has a diverse chemical biology (Figure 5).

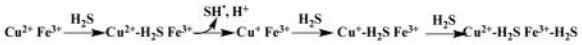
5a. H_2S and metal proteins

The physiological concentration and activity of H_2S can be mediated by binding to heme proteins. Such reactions can induce cytotoxic or cytoprotective responses in a concentration-

dependent manner as seen with mitochondrial cytochrome *c* oxidase [316, 317]. However, the interaction of H_2S with cytochrome *c* oxidase is complex as it can act as both inhibitor and electron donor [317, 318]. At low concentrations, H_2S can directly reduce the metal center. In the process, H_2S is oxidized to the thiyl radical or elemental sulfur, and O_2 is consumed (Eq. 23) [317, 319]. Elevated levels of H_2S can inhibit cytochrome *c* oxidase in a noncompetitive manner ($k_{on} = 1.5 \times 10^4\ M^{-1}\ s^{-1}$, $k_{off} = 6 \times 10^{-4}\ s^{-1}$) unlike NO, which is competitive with O_2 binding (Eq. 24) [319, 320].



23)



24)

The reaction of H_2S has also been studied extensively in hemoglobin and myoglobin. H_2S can reduce ferric heme, thus restoring O_2 binding function [319]. However, at higher concentrations, H_2S can react with oxyglobins to form sulfheme derivatives, which have weak affinities for O_2 [319, 321]. Formation of the sulfheme complex requires the presence of a histidine residue at the distal site [322, 323]. At physiological concentrations, the interactions of H_2S with hemeproteins such as cytochrome *c* oxidase, myoglobin and hemoglobin are suggested to be associated with activation of ATP-sensitive potassium channels and regulation of muscle relaxation [320, 324, 325].

5b. H_2S and O_2

O_2 is an antagonist of H_2S and plays a major role in H_2S bioavailability. Autoxidation, although spontaneous, is slow and leads to formation of sulfur (Eq. 25), which can then further react with excess sulfide to form polysulfides (Eq. 26).

$$2HS^- + O_2 \rightarrow 2S + 2OH^- \quad 25)$$

$$HS^- + xS \rightarrow HSS_x^- \quad 26)$$

The presence of free metals, phenols and aldehydes can accelerate this reaction [326, 327].

Similarly to thiols, H_2S also reacts with various ROS and RNS [328]. The reactions of H_2S with H_2O_2, $ONOO^-$ or alkyl peroxides to form hydrogen thioperoxide (HSOH; e.g., Eq. 27) [329] may be biologically relevant. HSOH is a reactive intermediate that can modify thiols or react with H_2S to form persulfides and polysulfides (Eq. 28), which are key players in H_2S-mediated signaling.

$$2HS^- + H_2O_2 \rightarrow HSOH + H_2O \quad 27)$$

$$HSOH + xHS^- \rightarrow HSS_x^- + H_2O \quad 28)$$

HSOH can also be further oxidized, for example to sulfite and sulfate. Such antioxidant properties of H_2S can downregulate several pro-inflammatory cytokines including NF-κB, TNF-α, IL-1β, IL-6 and IL-8 [330–332].

H_2S can undergo nitrosation (Eq. 29) or react with *S*-nitrosothiols (Eq. 30) to form the smallest nitrosothiol, HSNO [233, 333]. This reaction is not only associated with RNS scavenging but also is invoked as a possible mechanism for HNO biosynthesis as mentioned in Section 4.

$$HS^- + N_2O_3 \rightarrow HSNO + NO_2^- \quad 29)$$

$$HS^- + RSNO \rightarrow HSNO + RS^- \quad 30)$$

Interactions of H_2S with heme systems can indirectly control O_2 bioavailability, leading to reduced metabolic activity [334, 335]. The interplay of H_2S and O_2 can have widespread implications in the pathology of ischemia-reperfusion [336]. H_2S signaling may be pronounced in hypoxic environments such as tumors. Increased H_2S production from CBS in colorectal and ovarian cancers promotes proliferation, angiogenesis and migration, which can be reversed by silencing of CBS both *in vitro* and *in vivo* [337]. Cancer cells that survive hypoxic or oxidative damage show rapid cell proliferation and a nicotinamide phosphoribosyltransferase-dependent increase in tolerance to higher H_2S levels [338]. Thus treatment of cancer cells with an H_2S donor protects cells from drug-induced damage [338]. Inhibition of CBS may therefore be a potential area for development of anticancer therapeutics [337].

5c. H_2S and thiols

Fukuto and colleagues have hypothesized the formation of persulfides by the interaction of H_2S with oxidized thiols such as cystine (Eq. 31) [339].

$$RSSR + H_2S \rightarrow RSSH + RSH \quad 31)$$

Ida *et al*. developed a detection method that demonstrated the presence of persulfide and polysulfide species at substantially higher levels in mammalian cells, tissues, and plasma [340]. Miranda and Wink recently commented on the relationship between H_2S and persulfides, which are nucleophilic reductants that can modify protein structure and activity [341]. Polysulfides have emerged as a key class of reactive species in H_2S biology as they exhibit antibacterial, antifungal, antiviral and anticancer properties [342, 343].

H_2S can also react with key cysteine residues to undergo *S*-sulfhydration (-SSH) under physiological conditions. Many proteins including 20–25% of liver proteins, tubulin, actin

and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are sulfhydrated under physiological conditions, which may augment their activity. The role of protein sulfhydration in signaling has been reviewed by Paul *et al*. [344]. These findings open up a new aspect of sulfur redox biology that may impact thiol-based cellular signaling.

6. Carbon Monoxide and Hydrogen

CO is mainly formed by partial decomposition of organic matter, and similarly to H_2S, the toxic and environmental effects of CO have been extensively studied. On the other hand H_2 has no cellular cytotoxicity even at high concentrations. Biological H_2 is mainly produced by bacteria from hydrocarbons. Gut microbiome is rich in such bacteria. While these inorganic effector molecules are known to serve in various physiological and pathophysiological settings, [26, 345–347], their mechanisms of action are not fully understood at present.

6a. Carbon Monoxide

In the early 1950s, Sjöstrand showed for the first time that CO is produced under physiological conditions by degradation of hemoglobin [348, 349]. In the 1960s, Tenhunen and colleagues identified heme oxygenase as the endogenous source of CO [350, 351] (Figure 6).

Ryter *et al*. recently reviewed the biological effects of CO and its role in various biomedical applications, including protective effects in organ transplant, inflammatory pathways, cardiovascular function and cancer biology [14]. In addition, CO has also been implicated as a modulator of intraocular pressure and can play an important role in glaucoma therapy [352] and cardiac mitochondrial biogenesis [353].

Heme proteins are among the most important cellular targets of CO. For example, CO can bind to hemoglobin with an affinity ~250 times higher than O_2 [354]. Binding of CO to subunits of hemoglobin also increases the affinity for O_2 binding, thus inhibiting O_2 release. Even though this process is reversible, it can lead to CO poisoning [355, 356]. Another potential target of CO is the cytochrome P450 family of enzymes. P450s are involved in oxidative metabolism of drugs and xenobiotics. Physiological levels of CO are usually too low to inhibit P450s, however exogenous exposure to CO can lead to inhibition of P450s affecting drug metabolism and vascular tone [357]. Ferrous cytochrome *c* oxidase, which is a key respiratory chain enzyme, can also bind CO, and this may play a biological role under hypoxic conditions [358]. CO can also bind to and activate sGC similarly if less effectively than NO and induces vasorelaxation in rat-tail artery [359]; it also inhibits platelet aggregation [360].

6b. Hydrogen

Molecular hydrogen has long been considered as a viable option for renewable energy as water is the sole product of combustion of H_2. While certain microorganisms are able to metabolize H_2, the production of H_2 in human flatus was first reported in 1862 [361]. Levitt and colleagues showed in 1969 that more than 99% of H_2 production is colonic while the presence of excess small bowel bacteria increased H_2 production as well [362]. In 1982,

McKay and colleagues observed H_2 production by human intestinal anaerobic bacteria [363].

The role of H_2 in human physiology remains largely unexplored. In 2007 Ohsawa *et al*. reported that H_2 selectively reduced $^{\cdot}OH$ and $ONOO^-$ [364] although it does not scavenge radicals like NO and O_2^- that are involved in cellular signaling. Although facile, the reaction of $^{\cdot}OH$ with H_2 ($3.4 \times 10^7\ M^{-1}\ s^{-1}$) [365] may not be generally competitive with other pathways that consume $^{\cdot}OH$. However, this reaction prevents an immediate-type allergic reaction in mice, suggesting the possibility of selective modulation of signaling [366].

In 2010 Hong *et al.* reviewed the beneficial effects of exogenous H_2 in clinical and animal studies [367]. The observed antiapoptotic, antiinflammatory and antiallergy outcomes of consumption of H_2 saturated water or inhalation of H_2 were suggested to largely be a function of selective antioxidant effects. That H_2 diffuses rapidly and can cross the blood brain barrier may be beneficial in prevention of ROS-mediated diseases [368].

The combination of NO and H_2 significantly decreased cardiac infarct size and 3-nitrotyrosine formation compared to NO alone, and this effect was attributed to removal of RNS by H_2 [369]. Inhalation of H_2 by cardiomyopathic hamsters decreased oxidative stress and attenuated embryonic gene expression under hypoxic conditions [370]. This effect was also demonstrated in a rat model of myocardial ischemia-reperfusion injury [371]. H_2 also suppressed hepatic injury caused by ischemia/reperfusion [372].

In a mouse model lacking leptin receptors, exposure to H_2 induced fibroblast growth factor 21 (FGF21), which is involved in regulation of metabolism [373], This result indicates a role for H_2 in treatment of obesity and diabetes. H_2 may also serve as an effective and safe radioprotective agent [374]. Despite a growing literature on the protective role of H_2 in various conditions, the chemical biology of H_2 requires further investigation to understand the underlying mechanisms at a molecular level.

7. Crosstalk between Redox Signaling Molecules

The diverse physiological roles of O_2, NO, CO and H_2S pose key questions regarding their interaction with one another and the effect of such reactions on cell signaling. The reactions of O_2 with NO and H_2S have been described in earlier sections. While CO is not known to directly react with O_2, it can indirectly effect O_2-mediated cellular signaling. For example, HO-1 can modulate production of ROS from NADPH oxidase/NOX, suggesting that CO is involved in O_2 sensing [375] (Figure 7). NO can upregulate HO-1 expression thus increasing endogenous levels of CO, while CO can regulate iNOS expression and activity [376, 377]. As mentioned above, CO can also activate sGC leading to smooth muscle relaxation [378]. Based on their interrelationship and the similar roles of CO and NO, CO is speculated to substitute for NO under NO-deficient conditions and to regulate the bioavailability of NO.

Whiteman *et al*. showed that *in vitro* incubation of sodium hydrosulfide, an H_2S donor, with NO led to formation of RSNO [379]. On the other hand, H_2S can reduce GSNO to release NO [380]. Eberhardt *et al*. proposed that NO and H_2S can generate HNO resulting in

sustained calcium flux, release of calcitonin gene-related peptide (CGRP) and in turn vasodilation [381]. Recently, H_2S has been shown to inhibit iNOS expression via HO-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS) [331]. On the other hand, NO can bind tightly to the heme site of human CBS ($K_d \leq 0.23$ μM, $k_{on} = 8 \times 10^3$ M^{-1} s^{-1} and $k_{off} = 0.003$ s^{-1}) and regulate its activity *in vivo* [382]. Similarly, CO can react with the heme protein CBS, which is involved in H_2S production. Therefore, while CO may not play a direct role in biological processes, it has the ability to affect O_2, NO and H_2S dependent processes. Thus, crosstalk between these signaling agents can significantly impact physiological and pathophysiological processes.

8. Conclusions

The goal of this review is to provide an overview of biologically relevant reactions of the gaseous signaling molecules O_2, CO, NO, H_2S and H_2 and their interaction with each other to impact cellular signaling. These molecules have overlapping molecular targets including metalloproteins and thiols, which may lead to direct as well as indirect physiological and patho-physiological effects by modulating bioavailability. The biological roles of these signaling molecules are also both concentration and O_2-dependent. The implications of formation and regulation of these species in inflammatory pathways, cardiac pathophysiology and cancer biology is not fully understood. Further research to understand availability and interrelationships under physiological conditions may allow for development of novel therapeutic agents.

Acknowledgments

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Abbreviations

ALDH	aldehyde dehydrogenase
CGRP	calcitonin gene-related peptide
CO	carbon monoxide
COX	cyclooxygenase
CBS	cystathionine β-synthase
CSE	cystathionine γ-lyase
P450	cytochrome P450
N_2O_3	dinitrogen trioxide
N_2O_4	dinitrogen tetraoxide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide

$^{\cdot}OH$	hydroxyl radical
IPA/NO	isopropylamine diazeniumdiolate
LPS	lipopolysaccharide
LOX	lipoxygenase
MST	3-mercaptopyruvate-S-transferase
NOHA	N^{ω}-hydroxy-L-arginine
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
NO_2	nitrogen dioxide
HNO	nitroxyl
PARP	poly(ADP-ribose) polymerase
RNS	reactive nitrogen species
ROS	reactive oxygen species
STP	standard temperature and pressure
O_2^-	superoxide
SOD	superoxide dismutase
HSNO	thionitrous acid

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Highlight

Small inorganic molecules constitute an important class of signaling agent.

Regulated biosynthesis and kinetic constraints control signaling by these species.

Redox interchange is a key factor in signaling by inorganic effector molecules.

Different classes of inorganic molecules can interact to influence signaling.

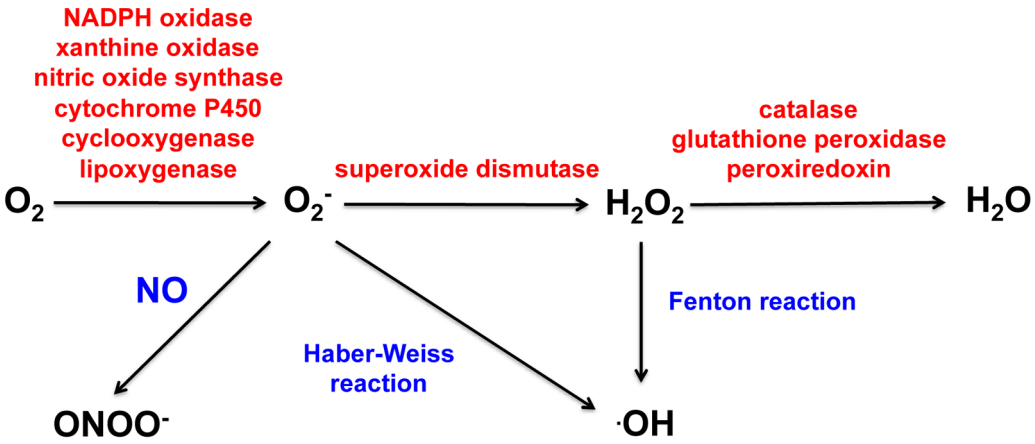

Figure 1.
Formation of physiologically relevant ROS by key enzymes and processes.

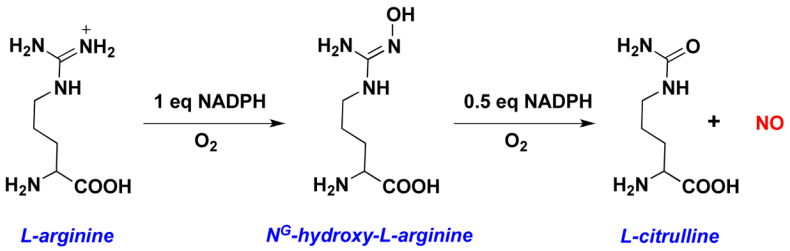

Figure 2.
Biosynthesis of NO from oxidation of L-arginine by nitric oxide synthase.

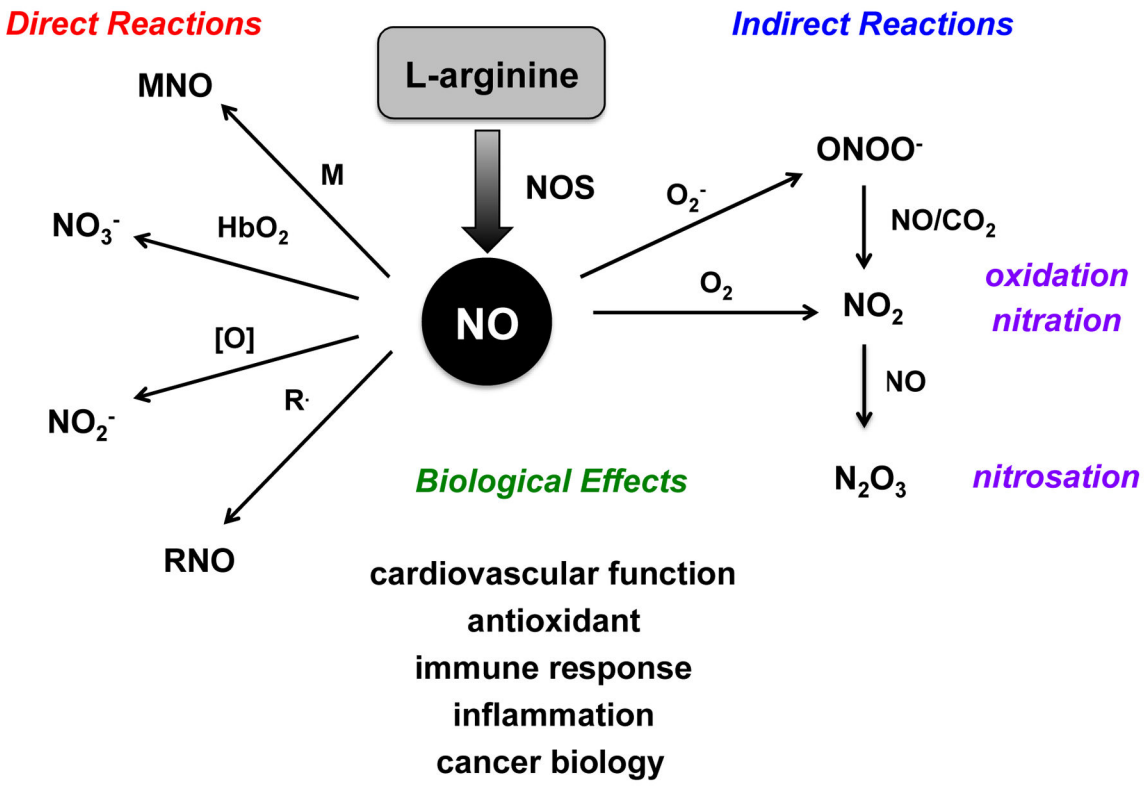

Figure 3.

The Chemical Biology of NO: direct and indirect reactions of NO with kinetically important cellular targets and their biological implications.

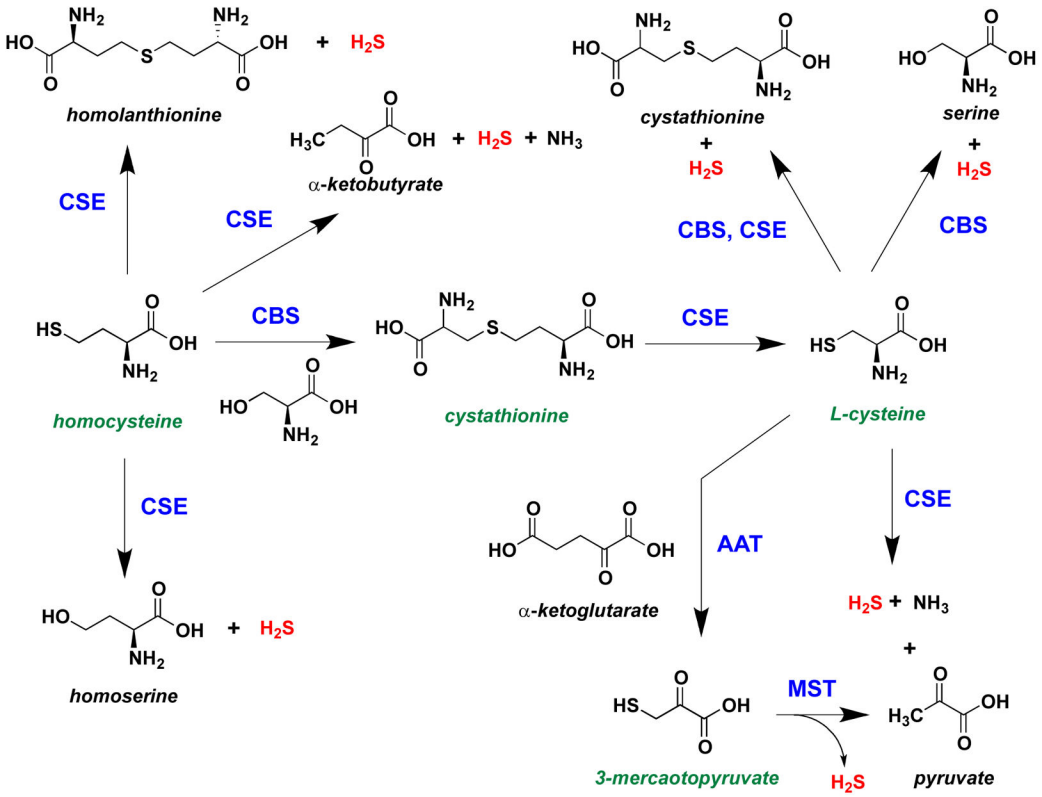

Figure 4.

Pathways for biosynthesis of H_2S from L-cysteine, homocysteine, cystathione and 3-mercaptopyruvate catalyzed by CBS, CSE and MST.

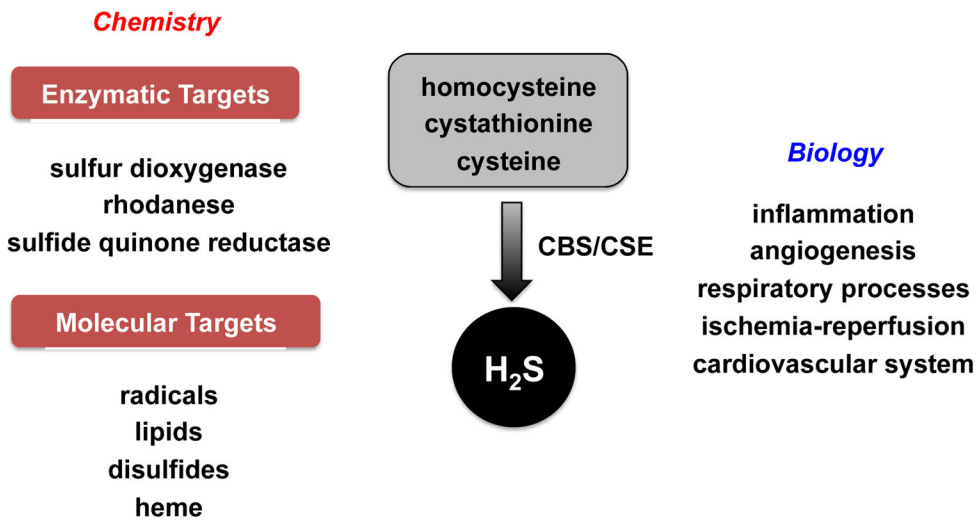

Figure 5.
Overview of the chemical biology of H_2S: biological targets of H_2S and their biological implications.

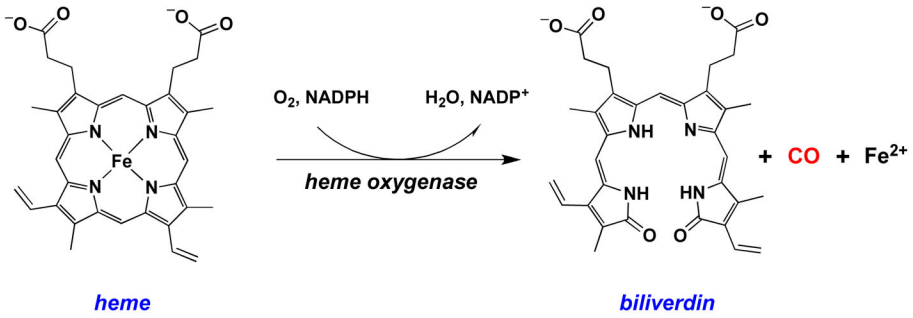

Figure 6.
Biosynthesis of CO from degradation of heme by heme oxygenase.

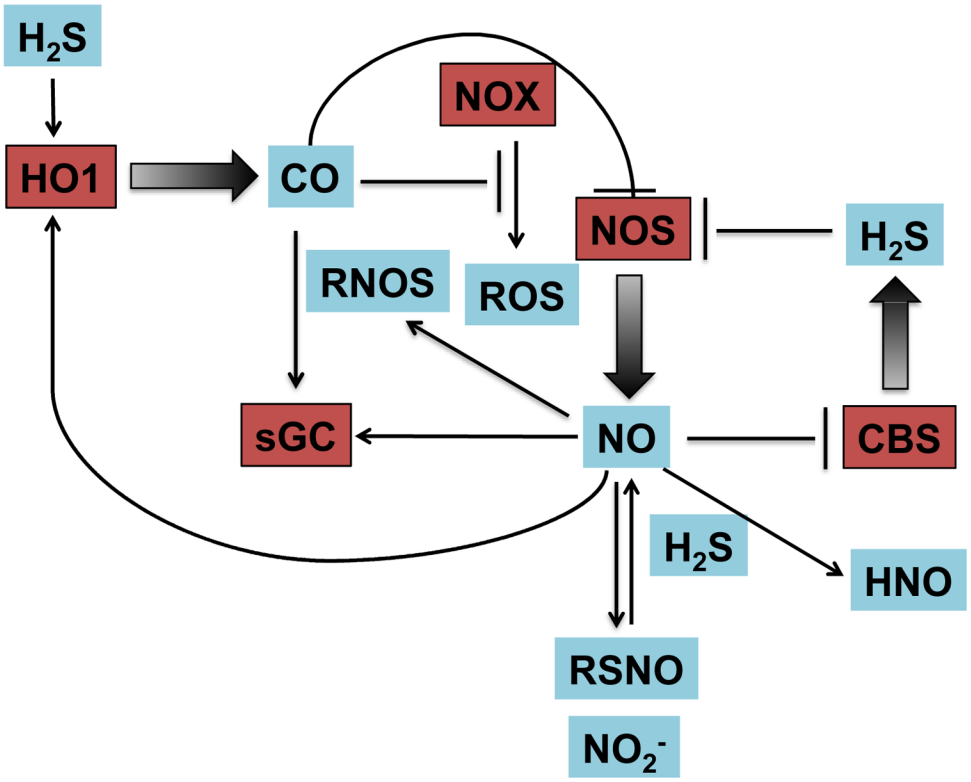

Figure 7.
Crosstalk and interdependence of inorganic signaling molecules.