

Gasotransmitters, poisons, and antimicrobials: it's a gas, gas, gas!

Mariana Tinajero-Trejo*, Helen E. Jesse* and Robert K. Poole*

Address: Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

* Corresponding author: Mariana Tinajero-Trejo (mbp09mt@sheffield.ac.uk); Helen E. Jesse (mbp09hej@sheffield.ac.uk); Robert K. Poole (r.poole@sheffield.ac.uk)

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Abstract

We review recent examples of the burgeoning literature on three gases that have major impacts in biology and microbiology. NO, CO and H₂S are now co-classified as endogenous gasotransmitters with profound effects on mammalian physiology and, potentially, major implications in therapeutic applications. All are well known to be toxic yet, at tiny concentrations in human and cell biology, play key signalling and regulatory functions. All may also be endogenously generated in microbes. NO and H₂S share the property of being biochemically detoxified, yet are beneficial in resisting the bactericidal properties of antibiotics. The mechanism underlying this protection is currently under debate. CO, in contrast, is not readily removed; mounting evidence shows that CO, and especially organic donor compounds that release the gas in biological environments, are themselves effective, novel antimicrobial agents.

Introduction and chronology

Gases play critical roles in life on earth. In microbiology, the best-known gases are nutrients or metabolites: dioxygen, carbon dioxide, dinitrogen, dihydrogen, methane and others [1]. This article highlights the extraordinary advances that have been made in the biology of three other gases – nitric oxide (nitrogen monoxide, NO), carbon monoxide (CO) and hydrogen sulfide (H₂S).

Great strides forward in understanding how and why these gases are important in mammalian physiology have kick-started a new area of “gasotransmitters”, i.e. small gaseous molecules that play key roles in biology, illustrated by a large number of reviews published in the last few years (see below). All these gases penetrate membranes, are poisons in excess, are endogenously generated and have important biological targets, especially metalloproteins [2-4]. The discovery in the 1980s that NO produced by mammalian NO synthases is important in the cardiovascular, immune and nervous systems [5-8] was the first impetus to this new area of biology [9]. A decade later, CO emerged as a neurotransmitter and regulator of the cardiovascular and immune systems [10-13]. Most

recently, H₂S has emerged as a third gasotransmitter, with key roles in the nervous and cardiovascular systems and regulation of cellular and whole body metabolism (reviewed in [14,15]). The literature on the biology of these three gases is now so vast that these and other reviews must act as a surrogate for the relevant papers: [16-20]. In the microbial world too, all three gases have important long-recognised roles: NO is an intermediate in denitrification [21] and is detoxified by pathogens [22,23], CO is an unusual carbon and energy source [24], and H₂S is well known to all microbiologists as a product of anoxic sulfate respiration [25]. Each gas is suggested to have valuable therapeutic applications [26,27].

Here, we present recent advances in the field that cement the view that each of these gases is not only toxic but also plays essential roles in metabolism. Toxicity has been most clearly harnessed by the innate immune response in the use of NO as a chemical weapon against pathogens, but may also find future applications via the therapeutic delivery of CO to pathogens. We look at the surprising evidence that NO and H₂S protect bacteria from the lethal effects of antibiotics. As a corollary, we report on two new

papers that challenge the premise that these gases act as antioxidants.

Lessons from mammalian physiology

The era of gas biology started with the recognition that NO was the elusive “endothelium-derived relaxing factor” (EDRF), a messenger (or gasotransmitter in current nomenclature) produced by endothelial cells that leads to vascular muscle relaxation. In 1998, this work led to the award of the Nobel Prize for Physiology or Medicine to Murad [28], Furchgott [8] and Ignarro [7]. A fourth major proponent of this idea, Salvador Moncada, was not so honoured. The story of these discoveries and the ensuing escalation of research on NO are now well known. NO is generated in man by a family of NO synthases (NOS, i.e. inducible NO synthase, endothelial NO synthase etc.) from arginine and has many critical roles in the cardiovascular, immune and central nervous systems. NO is the most chemically reactive of the gasotransmitters considered here and is a toxic radical: it is employed in the immune response of macrophages, constituting a key component of the arsenal of damaging species directed at engulfed pathogens [23]. Although Sjostrand provided experimental evidence for the endogenous production of CO in man in 1950, it was only in 1991 that Marks and others [10] hypothesised that CO might be another gasotransmitter, produced by haem oxygenases. Indeed, many of its physiological effects are similar to those of NO: roles in neurotransmission and platelet aggregation and anti-apoptotic activity are well documented. Nevertheless, CO is more notorious as the “silent killer”, while H₂S is more toxic to humans than HCN [3]. Less is known about H₂S, but it is synthesised endogenously and opens vascular smooth muscle channels [29].

All these compounds are gases at atmospheric pressure, all are more (H₂S) or less soluble in water and, being small and uncharged, move freely through membranes. No dedicated transporters are expected for the gases, but the nitrosating agent S-nitrosoglutathione (which is widely used experimentally as a source of “reactive nitrogen species”) is modified on transport through the periplasm and cytoplasmic membrane (Fig. 1). The jury is out on whether CO-releasing molecules must be transported inwards or can be expelled from cells. The sulfide anion that accumulates in the bacterial cytoplasm can be moved out by a specific transporter. Some of the salient physicochemical features of these gases and a comparison with oxygen are summarised in Table 1 and Fig. 1 shows their origins and effects.

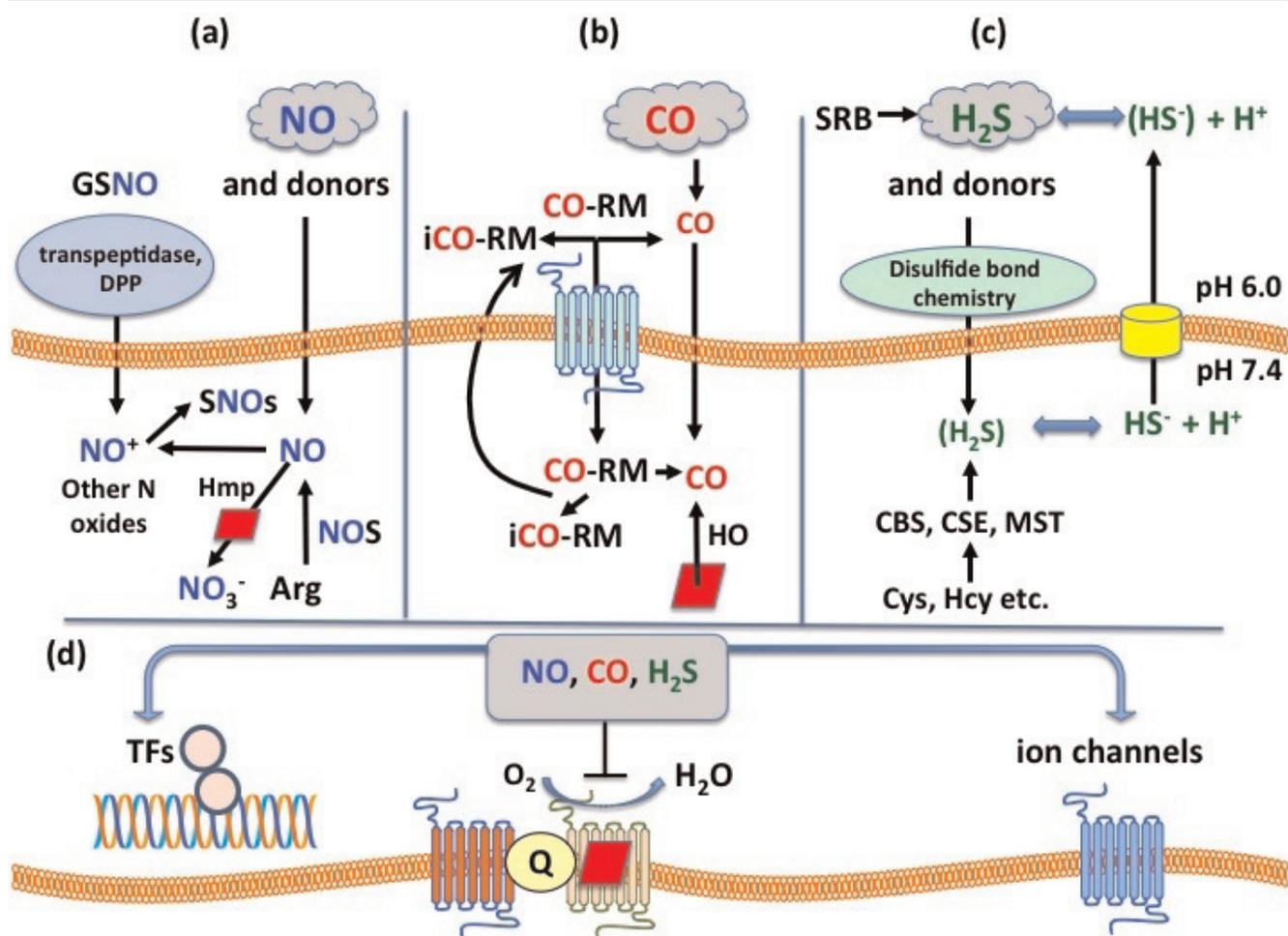
Nitric oxide – the old hand

Nitric oxide and reactive nitrogen species now occupy central positions in contemporary medicine, physiology,

biochemistry and microbiology. All these species are generated in biological systems from initial formation of NO (from nitrite, NO synthases, or other sources). The major targets of NO and reactive nitrogen species are metal centres (Table 1) and thiols, so that numerous critical biomolecules are at risk. Not surprisingly, microbes have evolved mechanisms for resisting or reversing such damage and many of these are critical for pathogenicity; such measures include the activities of hemoglobins that enzymically detoxify NO (to nitrate) [30], respiratory and NADH-linked NO reductases [31] and repair mechanisms (for example, those that reverse S-nitrosothiol formation) [32]. Microbial resistance to these stresses is generally inducible via the action of NO-sensing transcriptional regulators (such as Fnr, NorR, NsrR, NssR) [33,34]. However, a phenomenal growth in the literature on NO has often not gone hand in hand with an understanding of the intricacies and complexities of nitrogen chemistry. The distinctiveness of NO and its chemical cousins, nitrosonium (NO⁺), nitroxyl (NO⁻, HNO), peroxyxynitrite (ONOO⁻), nitrite (NO₂⁻) and nitrogen dioxide (NO₂) has been critically reviewed recently [22]. Unfortunately, some authors write “NO” when it is actually meant “in a generic sense”, thus confusing the biological literature. It should not be necessary to write “NO radical” to eliminate the possibility that one is actually meaning NO⁺ or some other congener. There is only one NO.

There is a growing literature on signalling functions for NO in microbes. An interesting example is the influence of NO on biofilm formation. Recently it has been reported that NO derived from periplasmic reductase positively influences biofilm formation in *Azospirillum brasilense* [35]. Conversely, NO donors aid in dispersing biofilms of *Pseudomonas aeruginosa* [36]. Recently, an NO sensor has been identified in *Vibrio fischeri*: H-NOX appears to be part of a signal transduction pathway to detect host-produced NO and influence iron utilisation genes [37,38].

NO is most efficiently detoxified in bacteria by the activity of haemoglobins that catalyse the conversion of NO and dioxygen to nitrate anion (Fig. 1). The best characterised such proteins are flavohaemoglobins but, even now, 15 years after this reaction was first proposed for the *Escherichia coli* protein [39], and 17 years since the finding that flavohaemoglobin gene expression was dramatically up-regulated by NO [40], there is still controversy about the reaction mechanism. Two recent reviews [41,42] assume the reaction to proceed via an NO dioxygenation mechanism in which oxygen binds to the ferrous haem, followed by NO. However, since 2001, Stamler’s group have favoured an alternative denitrosylase mechanism in which the protein binds NO during turnover *in situ*, and rapidly catalyses nitrate formation across a wide range of

Figure 1. Simplified schematic diagram of the origins of NO, CO and H₂S in bacteria and their major physiological effects

(a) NO accesses the cell interior via free passage through the membrane. The commonly used nitrosating agent, *S*-nitrosoglutathione (GSNO), a tripeptide, is hydrolysed in the periplasm and the nitrosated dipeptide transported inwards via a dipeptide permease (DPP) [32]. Intracellular NO is also generated by bacterial NO synthases (bNOS) [59] from arginine or anaerobically by nitrite reduction (not shown). NO or GSNO exhibit complex biological chemistry leading to the formation of various N oxides, especially *S*-nitrosothiols (SNOs) [22,32]. The best understood route for NO detoxification is a dioxygenase or denitrosylase reaction with oxygen catalysed by the flavohaemoglobin Hmp [22].

(b) CO accesses the cell interior via free passage through the membrane. CO-releasing molecules (CO-RMs) may or may not require transport systems. Release of CO from CO-RMs yields an “inactive” form (iCO-RM) either inside or outside the cell, the biology of which needs careful consideration [81]. The fate of CO-RM or iCO-RM is unknown but efflux systems might operate. CO is also generated endogenously in certain bacteria by haem oxygenases (HO) from the breakdown of haem (red diamonds).

(c) H₂S accesses the cell interior via free passage through the membrane but the hydrosulfide anion, which is prominent intracellularly by virtue of the pH gradient, may be exported by a specific transporter (yellow) [91]. Three endogenous mechanisms for H₂S generation from Hcy and Cys have been identified [99].

(d) Global consequences of the three gases include activation of gas-specific transcription factors (TFs), inhibition of respiratory oxygen reduction by binding to the haem(s) of terminal oxidases, and modulation of bacterial ion transport [77]. Q, quinones involved in respiration.

oxygen concentrations [43]. These alternative views are debated in letters [44,45] that promptly followed the Forrester review [42]; however, it is not disputed that flavohaemoglobins *do* catalyse the rapid detoxification of NO to nitrate and play a critical role in the response of pathogenic microorganisms to the innate immune system.

An interesting computational approach to the complexity of NO biochemical networks has just been published [46] and concluded that Hmp is the dominant NO-consuming pathway at oxygen concentrations down to about 35 μM but, interestingly, loses effectiveness as NO delivery rates increase.

Table 1. Physicochemical properties of oxygen and three gasotransmitters

| | Oxygen, O ₂ | Carbon monoxide, CO | Nitric oxide, NO | Hydrogen sulfide, H ₂ S* |
|---|---|--------------------------|--|---|
| Molar mass, g/mol | 32 | 28 | 30 | 34 |
| Solubility in water, 20 °C | 0.0043 | 0.0028 | 0.0062 | 0.40 |
| Molecular size (pm) | 121 | 113 | 115 | 134 |
| Importance of redox metabolism in biology | +++ | + | ++ (radical) | ++ |
| Haem ligand? | yes | yes | yes | yes |
| Metal binding | +++ | +++ | +++ | +++ |
| Major mechanisms of endogenous generation | Catalase, photosynthesis | Haem oxygenases | NO synthases, nitrate/nitrite respiration, from SNOs (minor) | CBS, CSE, MST, sulfate reduction |
| Important roles in microbes | Major biological oxidant and co-substrate | Carbon and energy source | Intermediate in denitrification, antibiotic resistance | Metabolic intermediate, antibiotic resistance |

* pK_a is 6.8, so HS⁻ is dominant species in biology

Even though (almost) all haemoglobins (flavo-haemoglobins, single-domain and truncated globins) seem to possess NO-consuming activity, this physiological function *in vivo* has been demonstrated for only a few globins. Indeed, of the very large number of globin sequences identified in prokaryotic genomes (c. 1161 globin-containing genomes), only a handful has been subjected to rigorous genetic and physiological characterisation and the vast majority of the evidence pertains to flavohaemoglobins [47]. The most notable exception is the single-domain globin of *Campylobacter jejuni*, a myoglobin-like protein that is expressed in bacteria on challenge with NO, and mutation of which elicits an NO-sensitive phenotype [47,48]. The true function of most microbial globins is unknown [47].

Although many globin crystal structures from all classes are available (at least 15 bacterial and archaeal globins), understanding globin function(s) has not been greatly facilitated by these structures or by the numerous elegant kinetic experiments on ligand binding to the purified proteins. For example, the structure of the second (truncated) haemoglobin from *C. jejuni* has been solved [49-51] and its oxygen affinity is remarkably high [52], but we still do not understand its role *in vivo*.

The expression of globins in a convenient heterologous host is often exploited to test functional properties when genetic manipulation of the native host is difficult. An especially useful model is the flavohaemoglobin-lacking *E. coli* (*hmp* mutant), which is hypersensitive to NO. When complementation of the phenotype is observed, it is generally interpreted to demonstrate detoxification properties for the heterologously expressed globin (for a recent example, see [53]). However, the discovery of a function in a heterologous organism does not necessarily tell us that the protein will have the same function *in the native host*, especially when the bacteria are phylogenetically distant. For example, the requirement for interaction with

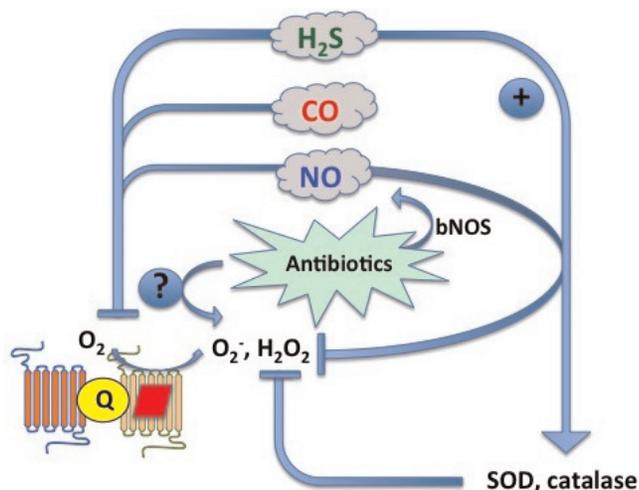
a cognate reductase by single-domain or truncated globins (as in [53]) and/or for oxygen for the NO → NO₃⁻ reaction may be accomplished in *E. coli* but not in the native host (reviewed in [47]).

Although generally harmful, NO may have beneficial effects, as in the case of the squid-*Vibrio* light-organ symbiosis, where NO serves as a signal, antioxidant and specificity determinant [54-56]. A recent high-profile case concerns the NO generated endogenously by certain bacteria from arginine by the activity of bacterial NO synthase (Figs. 1,2). Two papers reported that this NO protects bacteria against oxidative stress [57,58]. Later, many antibacterial agents were shown to suppress growth of a *nos* mutant lacking bacterial NO synthase [59]. The authors suggest that NO-mediated antibiotic resistance is explained in two ways: first, by chemical modification of the toxic compounds (for example, the nitrosation by NO⁺ of aromatic amino groups on acriflavine) and, second, by NO-protecting bacterial cells against the oxidative stress that antibiotics like quinolones generate (Fig. 2). However, two very recent papers in *Science* question the premise that killing by bactericidal antibiotics depends on reactive oxygen species (see below).

Carbon monoxide – the new NO?

CO is among the most abundant air pollutants in developed countries and its dangers are compounded by difficulties in detecting it. Tragic cases of CO fatalities are frequent in the media, including one recently involving many street children in China (<http://www.bbc.co.uk/news/world-asia-china-20389004>). Nevertheless, the ability of animals, plants and pathogenic microbes to make CO via haem oxygenase enzymes is widespread and the CO generated has potent *beneficial* biological consequences [60]. CO is now identified as a “gasotransmitter” with wide-ranging effects on vasodilation and inflammation [10-12]. The emerging view is that CO and CO-releasing molecules have biological effects

Figure 2. Proposed interactions between NO, CO and H₂S with antibiotics and oxidative stress in bacteria



All three gases are potent inhibitors of terminal oxidase activity in bacteria, leading to the accumulation of ROS (reactive oxygen species), initially superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). ROS are detoxified by superoxide dismutases (SOD) and catalases. ROS may also be generated by chemically unrelated antibiotics [102], although this is now disputed [104,105]. Antibiotics may also increase NO formation from bNOS [59] while H₂S induces activity of SOD and catalase.

far-removed from the classical view that CO is a toxic gas, useful to haem biochemists, but merely a by-product of haem oxygenase-catalysed haem degradation.

In certain bacteria, CO is an oxidisable substrate and must be sensed. In *Rhodospirillum rubrum* and *Carboxydotherrmus hydrogenoformus* [61], the *coo* operon encodes a CO dehydrogenase, which allows the anaerobic metabolism of CO. The expression of this operon is regulated by the CO sensor CooA [62], a member of the CRP/FNR family. In response to a reducing cellular environment, CO binds to ferrous haem of CooA, causing a conformational change that permits DNA binding and therefore transcription of the *coo* operon [63,64]. Recently, an ingenious use of CooA has been described in which the natural sensitivity of CooA to CO has been exploited in a sensitive fluorescent protein reporter for CO imaging in living cells [65]. However, homologues of this canonical CO oxidation system, including CooA, CO dehydrogenase, and a CO-dependent Coo hydrogenase, are also present in the sulfate-reducing bacterium *Desulfovibrio vulgaris*, although it grows only poorly on CO. Recent global transcriptional analyses suggests that CooA and CO dehydrogenase are used during normal metabolism of this bacterium [66]. Another haem-containing CO sensor, RcoM from *Burkholderia xenovorans*, which is also able to bind NO,

regulates the expression of genes in response to the redox state of the cell [67,68].

CO also has useful antibacterial features. It enhances phagocytosis of *E. coli* [69] by macrophages and shows promising effects on bacteraemia in sepsis [12,70]. Because of the difficulties of studying CO in the laboratory and using it therapeutically (see, however, [71]), CO-releasing molecules, mostly metal carbonyl compounds, developed decades ago by chemists, have more recently been used in biological studies [72]. These release CO in biological milieu with defined kinetics and stoichiometry, promoted by reaction with other species [73] or enzymic or photochemical activation [74]. CORM-3 (Ru(CO)₃Cl (glycinate)) is a water-soluble CO-releasing molecule that has numerous beneficial effects in mammalian systems and is an effective antibacterial agent [75]. CORM-3 decreased bacterial counts in the spleen and increased survival of mice following experimental bacteraemia [76]; injection into mice increased phagocytosis of *Enterococcus faecalis* and rescued haem oxygenase-deficient mice from sepsis-induced lethality [70]. Thus, therapies involving haem oxygenases, CO or CO-releasing molecules have great potential for treating infections [12], but the microbiological literature on these compounds is very limited and only a handful of the hundreds of CO-releasing molecules made over the past few years have been tested against microbes. At the time of writing, for example, no papers on the effects on microbes of any photo-CORM, which releases CO on demand after photolysis, have appeared. Our rudimentary knowledge of the modes of actions of CO and CO-releasing molecules, and the difficulty of drawing conclusions from so few CO-releasing molecules limits progress.

In many cases, the antimicrobial effects of CO-releasing molecules are clearly caused by the released CO, as control molecules that do not release CO do not induce these effects [75,77]. However, there is mounting evidence that CO-releasing molecules are much more powerful antibacterial agents than CO [75,78] and elicit biological effects beyond those of CO gas. For example, CO-releasing molecules exhibit antimicrobial effects even under highly aerobic conditions where CO gas is ineffective (because the gas is a competitive inhibitor with oxygen) [75]. Furthermore, a recent study of the effects of CORM-3 on the respiration of a range of bacteria found that this CORM elicited in *E. coli* transient stimulation of respiration, prior to respiratory inhibition; these effects were not mimicked by CO gas [77]. Uncoupling of respiration by promotion of proton flux, as proposed in mitochondria [79,80], was ruled out. In *E. coli*, the working hypothesis is that CO-releasing molecules cause the opening of other ion channels (K⁺,Na⁺), which transiently collapses the

protonmotive force and disturbs ion transport. The reason that CO-releasing molecules potentiate the effects of CO may be that they deliver CO directly into bacterial cells, thereby allowing a high concentration of this gas to accumulate at the sites of action [75] – the “Trojan Horse” mechanism [77].

As described above, CO is a classical inhibitor of respiration in both eukaryotes and prokaryotes, and CO from CO-releasing molecules shares this property. CO is delivered from CO-releasing molecules directly to the haems of cytochromes *bd* and *bo'* of the aerobic respiratory chain of *E. coli* and transcriptomic profiling has shown that exposure of *E. coli* to CORM-3 results in down-regulation of the *cyo* genes encoding cytochrome *bo'* and slight up-regulation of the *cydAB* genes encoding cytochrome *bd-I* [75], suggesting a role for cytochrome *bd-I* in resisting CORM-3. Subsequent time-resolved analysis revealed that the transcription of *appBC*, which encodes cytochrome *bd-II* is not affected in response to CORM-3 [81]. In support, a strain expressing cytochrome *bd-I* as the sole oxidase is less susceptible to both respiratory and growth inhibition by this compound [82], consistent with the established role of this oxidase in resisting a variety of environmental stresses including NO [83]. In the case of NO, it has been proposed that cytochrome *bd* resists inhibition because of an exceptionally high off rate (k_{off} 0.163 s^{-1} for cytochrome *bd*, compared to 0.03 s^{-1} for cytochrome *bo'* [83]), although it is difficult to draw such conclusions for CO due to discrepancies in the literature as to the off rate of this gas from cytochrome *bd-I* (see references in [82]).

CO is implicated in many recent studies as an antioxidant, for example in ischemia-reperfusion injury *in vitro* [84]. On the other hand, because CO is a potent inhibitor of respiration, it may increase generation of reactive oxygen species in mitochondria [85,86] or bacteria [87] (Fig. 2). One recent study [88] suggests that CORM-2 promotes the formation of reactive radical species, even in the absence of cells, and that such species are largely responsible for CORM-mediated bacterial killing. This assertion appears to be supported by the oft reported, but poorly understood, observation that N-acetylcysteine protects against CORM-2 and CORM-3 toxicity (e.g. [76,89]). Other data, however, contradict this view [81]; some antioxidants do not share this property and N-acetylcysteine is effective at inhibiting CO-RM uptake by *E. coli*, presenting us with a new testable hypothesis for the protective effects of N-acetylcysteine [82]. The field is in a state of flux and further insights are anticipated but, unlike the case for NO (above) and H₂S (below), there appears to be no clear evidence yet that CO or CO-releasing molecules either protect against antibiotics or potentiate their activities.

Hydrogen sulfide – an (un)welcome gate crasher?

Hydrogen sulfide is a toxic, freely permeable, malodorous gas. It is very soluble in water (Table 1) and the equilibrium between its three states (H₂S, HS⁻ and S²⁻) is pH-dependent. Here, we write “H₂S” or “sulfide” (HS⁻) while recognising the importance of pH in controlling speciation [90]. At 37 °C the pKa is 6.76; since the pH of the human large intestine is in the range 5.5-7.0, H₂S and HS⁻ are the predominant sulfide species, and the hydro-sulfide anion (HS⁻) will be the dominant species inside gut bacteria [91]. An exporter for this anion has been identified in *Clostridium difficile* [91]. Recently, H₂S has been recognized as an important gasotransmitter in higher organisms (reviewed by [15,92,93]). This gas is abundant in the anaerobic lumen of the gut (1-2 mM total sulfide) with ~60 μM being present in a free, unbound form where it is generated by the reduction of sulfate and the decomposition of sulfur-containing organic compounds, such as cysteine, by resident microflora. Sulfate-reducing bacteria generate H₂S by the reduction of various sulfur compounds, e.g. SO₄²⁻ or S⁰ under anaerobic conditions, often using H₂ or simple organic molecules as an electron donor. H₂S and SO₄²⁻ are also generated by bacterial disproportionation of thiosulfate. Furthermore, human faecal slurries ferment cysteine, (via the action of cysteine desulfurases, which yield H₂S, ammonia and pyruvate) and other organic sulfur compounds [94]. Dietary polysulfides from “healthy” sources (garlic, onions etc.) further increase formation of H₂S. Thus, H₂S is abundant in the anaerobic environment of the mammalian lower intestine and consequently the gut epithelium is exposed to this potent signalling molecule and toxin.

Exogenous H₂S modulates the behaviour of intestinal epithelium cells [95]; however, the effects of exposure to exogenous H₂S are considered less significant than those elicited by endogenous H₂S production by the enzymes cystathionine-β-synthase and cystathionine-γ-lyase [92,96]. Endogenous H₂S levels are uncertain because of extraction and binding issues, but tissue homogenates and other material produce 1-10 μM of free H₂S in the presence of cysteine and low O₂. These significant concentrations control the relaxation of blood vessels, inhibit inflammation, and modulate neuronal activity [92]. The precise molecular mechanisms by which H₂S elicits these positive effects are not well established. However, the thiol groups of protein cysteine residues are modified by H₂S yielding hydropersulfide moieties (S-sulfhydration), which can result in altered activity. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity is enhanced seven-fold by S-sulfhydration of Cys-150 [97]. Thus, S-sulfhydration is an important post-translational modification in higher organisms, with implications for

protein function and transduction of the H₂S signal into altered behaviour.

As well as acting as an important signalling molecule, H₂S damages cell components and mediates its toxic effects, at least in part, by inhibiting respiration, reacting with other metals and by reducing protein disulfide bonds. Terminal respiratory oxidases have been known to be inhibited by H₂S for many decades [2]. Although the significance of H₂S signalling in higher organisms is now established, essentially nothing is known about the effect of H₂S on gut bacteria that are exposed to fluctuating concentrations of exogenous H₂S in their preferred niche and/or that generate endogenous H₂S [98]. The major source of endogenous H₂S production by *E. coli*, identified by Shatalin *et al.* [99], was the combined action of the L-cysteine transaminase (AspC) and the 3-mercaptopyruvate sulfurtransferase SseA (MST). However, there are several other potential H₂S-generating enzymes in *E. coli*, including CysJI, MetC, TnaA and H₂S-consuming enzymes, e.g. CysEK and CysM. In other bacteria like *Bacillus anthracis*, *Staphylococcus aureus* and *P. aeruginosa*, the main mechanisms are via cystathionine-β-synthase/cystathionine-γ-lyase [99]. We know much less about potential resistance mechanisms for H₂S in bacteria, but plant pathogenic bacteria use the *bigR* operon for H₂S detoxification via the action of a sulfur dioxygenase (Blh) and a sulfite exporter [100].

Emerging information on the biological roles of H₂S is likely to have significant impact in the foreseeable future, following the demonstration [99] that H₂S protects bacteria from a diversity of antibiotics by mitigating the effects of oxidative stress. Note, however, that this contrasts with a previous report that H₂S *potentiates* hydrogen peroxide-induced killing of *E. coli* [101]. The suggestion that H₂S can act as “*a universal defence against antibiotics in bacteria*” [99] is predicated on the assumption, first, that H₂S is an antioxidant and, second, that numerous antibiotics exert their bactericidal activities by mitigating oxidative stress. Let us now examine the second of these.

Gas interactions with microbes, antibiotics and other antimicrobial agents - do we really know how antibiotics work?

The papers cited above from Shatalin [58,99], Gusarov [57,59] and others suggest that, separately, NO and H₂S production may constitute a natural bacterial defence method against antimicrobial compounds. The specific proposal is that these gases (but not CO, yet) partially neutralise the effectiveness of antibiotics by suppressing the oxidative stress that antibiotics induce. This model of antibiotic action [102,103] appears to be a unified explanation of the effectiveness of numerous bactericidal

compounds that all induce the formation of reactive oxygen species by inhibiting the electron transport chain. However, this model fails to explain many aspects of antibiotic action, such as the effectiveness of bactericidal antibiotics against *Streptococcus pneumoniae*, which lacks any electron transport chain. Recently, Keren *et al.* [104] have produced persuasive evidence that a cell's probability of survival in the presence of an antibiotic does not correlate with the level of reactive oxygen species. Furthermore, thiourea, a reagent that quenches reactive oxygen species, is equally effective at protecting cells from antibiotics under anaerobic conditions, when reactive oxygen species cannot be formed. Likewise, Liu and Imlay [105] show that antibiotic treatment does not promote hydrogen peroxide generation or elevate intracellular free iron, a precursor to reactive oxygen species-induced damage via Fenton chemistry.

Perhaps curiously, neither of the most recent *Science* papers [104,105] mention the implications of their findings for the protection by NO and H₂S against bactericidal antibiotics. We are therefore left with puzzles: what is the mechanism of gas-mediated antibiotic resistance, does CO share this remarkable property and can the clinical effectiveness of antibiotics be modulated by endogenous microbial gas production? Only time will tell.

Questions, controversies, and conflicts

There is no doubt that NO, CO and H₂S play key roles in bacterial life, survival strategies and metabolism, undreamt of a few decades ago. Despite the rapid progress on all three gases in the mammalian field, only NO has achieved a degree of prominence in microbiology as a gasotransmitter or signalling molecule. CO and H₂S deserve more attention and the possible link between the latter and antibiotic tolerance should kick-start such activity. In the case of CO, all the evidence points at present to its potential as an antimicrobial agent, rather than a protective molecule for bacteria. In fact, we are unaware that antibiotic tolerance elicited by CO has ever been reported. CO and other products of haem degradation are considered as antioxidants in mammalian systems, but it remains to be seen whether CO can exert such a role in bacteria, or even whether the concept that antibiotics kill bacteria by stimulating reactive oxygen species formation withstands further careful scrutiny.

Since NO and H₂S appear to act as bacterial defences against antibiotics, future therapeutic strategies might involve targeting the gas-generating enzymes – bacterial NO synthase [58,59,106] and the three H₂S-synthesising enzymes identified thus far [99]. In the case of CO, a more productive strategy may be to develop clinically effective and permissible CO-releasing molecules. The most

commonly used, CORM-2 and CORM-3, are both ruthenium metal carbonyls and there is likely to be resistance to using a “non-biological” delivery molecule; on the other hand, witness the widespread use of cis-diamminedichloroplatinum(II) (Cisplatin), a chemotherapy drug that is given to treat testicular, bladder, and other cancers.

Despite an accelerating interest from microbiologists in these three remarkable gases, we urgently need more fundamental research into their synthesis, functions, and potential roles in therapy against pathogenic microbes.

Abbreviations

CORM-2, tricarbonyldichloroRu(II) dimer; CORM-3, Ru(CO)₃Cl(glycinate); DPP, dipeptide permease; EDF, endothelial-derived relaxing factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, S-nitrosoglutathione; Hcy, homocysteine; iCO-RM, “inactive” form of CO-RM after CO release; MST, 3-mercaptopyruvate sulfurtransferase; Q, quinones involved in respiration; SNOs, S-nitrosothiols; SOD, superoxide dismutases; TFs, transcription factors.

Disclosure

The authors declare that they have no disclosures.

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