

REVIEW ARTICLE

Effects of nitrogen monoxide and carbon monoxide on molecular and cellular iron metabolism: mirror-image effector molecules that target ironRalph N. WATTS*, Prem PONKA† and Des R. RICHARDSON*¹

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Many effector functions of nitrogen monoxide (NO) and carbon monoxide (CO) are mediated through their high-affinity for iron (Fe). In this review, the roles of NO and CO are examined in terms of their effects on the molecular and cellular mechanisms involved in Fe metabolism. Both NO and CO avidly form complexes with a plethora of Fe-containing molecules. The generation of NO and CO is mediated by the nitric oxide synthase and haem oxygenase (HO) families of enzymes respectively. The effects of NO on Fe metabolism have been well characterized, whereas knowledge of the effects of CO remains within its infancy. In terms of the role of NO in Fe metabolism, one of the best characterized interactions includes its effect on the iron regulatory proteins. These molecules are mRNA-binding proteins that control the expression of the transferrin receptor 1 and ferritin, molecules that are involved in Fe uptake and storage

respectively. Apart from this, activated macrophages impart their cytotoxic activity by generating NO, which results in marked Fe mobilization from tumour-cell targets. This deprives the cell of the Fe that is required for DNA synthesis and energy production. Considering that HO degrades haem, resulting in the release of CO, Fe(II) and biliverdin, it is suggested that a CO–Fe complex will form. This may account for the rapid Fe mobilization observed from macrophages after haemoglobin catabolism. Intriguingly, overexpression of HO results in cellular Fe mobilization, suggesting that CO has a similar effect to NO on Fe trafficking. Preliminary evidence suggests that, like NO, CO plays important roles in Fe metabolism.

Key words: carbon monoxide, iron, nitric oxide, nitrogen monoxide.

GENERAL INTRODUCTION

The role of iron (Fe) in the active site of a number of critical molecules such as ribonucleotide reductase, cytochromes and [Fe-S]-cluster-containing proteins is well known [1,2]. Fe also acts as a target for both nitrogen monoxide (NO) and carbon monoxide (CO), two messenger molecules with expanding roles in a plethora of biological functions [3–6]. Over the last decade it has become clear that NO produced by nitric oxide synthase (NOS) and CO generated by haem oxygenase (HO), are vital physiological effectors [3,4]. These small diatomic molecules play important roles in a variety of processes, including neurotransmission, the immune response, vascular tone, the control of respiration, inhibition of platelet aggregation, and the suppression of hypertension [3,7–15].

Both NO and CO have a high affinity for Fe, and act as ligands to complete the co-ordination shell of this atom. Indeed, the interaction of NO and CO with transition metals, particularly Fe, is a well-known branch of co-ordination chemistry [16] and mediates many of their biological effects. For instance, the earliest records of the CO–Fe interaction came from studies on the role of CO in asphyxia, where it was found to bind Fe in the haem moiety of haemoglobin (Hb) [17]. More recently, NO, and, to a much lesser extent, CO, have been shown to affect the intracellular metabolism of Fe via their ability to directly bind this metal ion [18–20]. In the current review we examine what is known concerning the interactions of NO and CO with Fe and the subsequent effects on cellular metabolism.

BIOLOGICALLY RELEVANT PROPERTIES AND REACTIONS OF CO AND NO

Some of the biologically significant properties of NO and CO are listed in Table 1. As noted above, both have a high affinity for Fe. However, while both CO and NO bind Fe(II) avidly in haemoproteins, CO, unlike NO, does not bind to Fe(III) haemoproteins [21].

In contrast with CO, which exists as a stable neutral molecule, NO can be found in distinct redox-related states (Table 1) that have different reaction specificities. These forms of NO include nitric oxide (NO[•]), nitroxyl anion (NO[−]) and the nitrosonium ion (NO⁺) [22]. Apart from the reaction with Fe and other transition metals, NO can react with oxygen, superoxide and thiol groups [22]. Unlike CO, which has a long half-life [23], the biological half-life of NO[•] is in the order of seconds (Table 1), and its reaction with superoxide generates peroxynitrite (ONOO[−]). This latter species is highly reactive and rapidly decomposes to result in the cytotoxic hydroxyl radical (OH[•]).

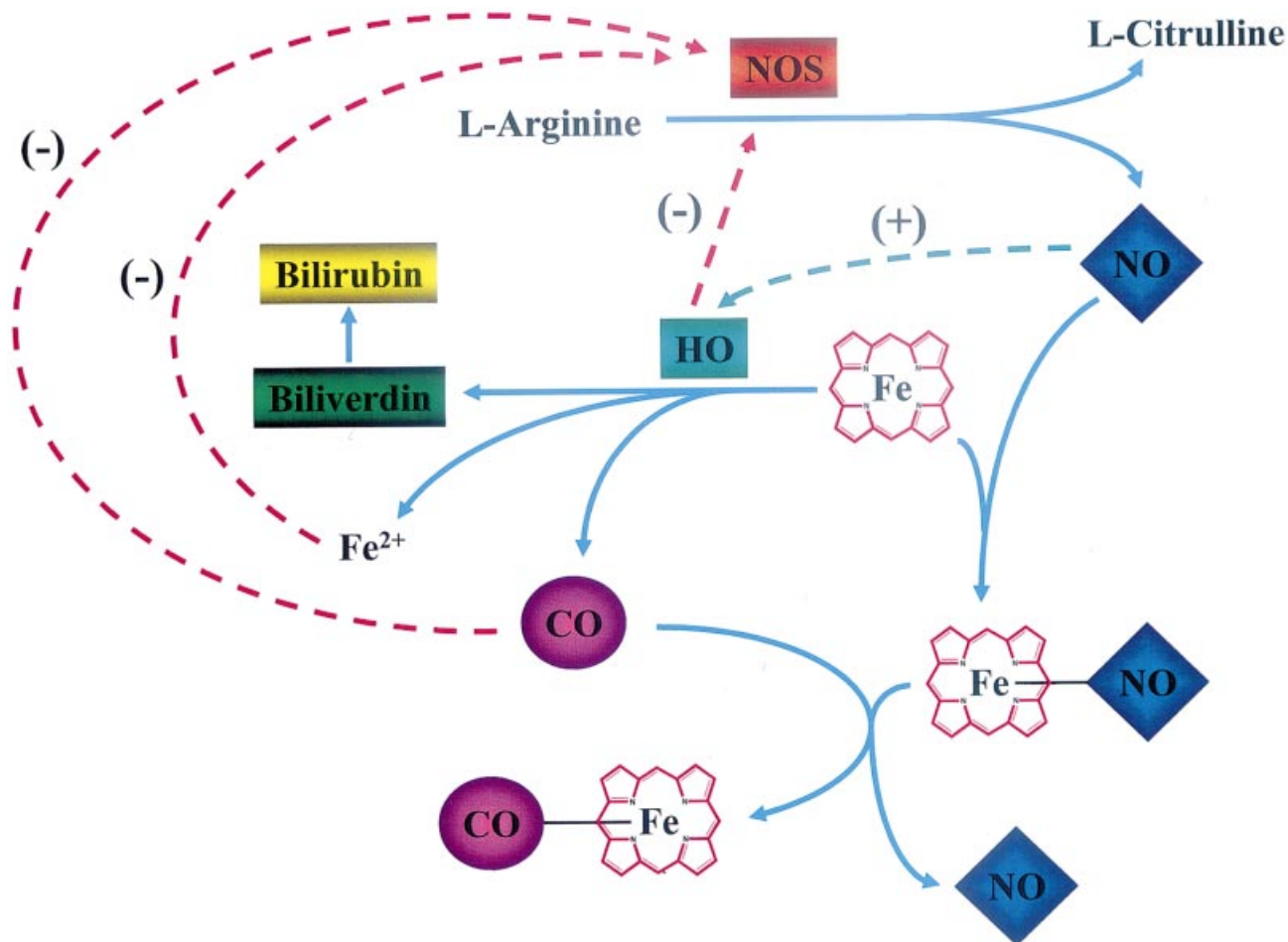
The role of the nitroxyl anion (NO[−]) in physiological systems remains unclear, although some researchers have suggested that it may be a product of NOS activity [24]. On the other hand, the role of NO⁺ in biology is well documented. Studies by Stamler [22] and others have suggested that NO⁺ can S-nitrosylate critical thiol groups in a variety of molecules, such as glyceraldehyde-3-phosphate dehydrogenase [26], Hb [27], the *N*-methyl-D-aspartate ('NMDA') receptor subunits 1 and 2 [28,29], the retinoblastoma susceptibility gene product ('pRb') [29,30], ornithine de-

Abbreviations used: eNOS, endothelial nitric oxide synthase; GSNO, S-nitrosoglutathione; HO, haem oxygenase; IFN- γ , interferon- γ ; iNOS, inducible NOS; IRE, iron-responsive element; IRPs, iron-regulatory proteins; LPS, lipopolysaccharide; mtNOS, mitochondrial NOS; nNOS, neuronal NOS; Nramp2, natural resistance-associated macrophage protein-2; RE, reticuloendothelial; sGC, soluble guanylate cyclase; SIN-1, 3-morpholino-sydonimine hydrochloride; SNP, sodium nitroprusside; Tf, transferrin; TfR1 and TfR2, transferrin receptors 1 and 2; UTR, untranslated region.

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Table 1 Summary of the chemical and biological properties of NO and CO and their effects on some biological targets

Property or action	CO	NO	Reference(s)
M_r	28.01	30.01	[117]
Solubility in water at 20 °C (ml/100 ml)	2.3	4.6	[117]
Radical species	None	NO^\cdot , NO^+ , NO^-	[22,117]
Physiological lifespan	Stable ($t_{1/2,\text{COHb}} = 36\text{--}137$ min)	0.025–12 s	[22,100,190–194]
Physiological synthesis	HO	NOS	[25,29,80,81,82,94,95]
	HO-1 inducible HO-2 constitutive HO-3 constitutive Fe/lipid peroxidation	nNOS constitutive iNOS inducible eNOS constitutive mtNOS constitutive	
Physiological production ($\mu\text{mol/day}$)	500	850	[117]
Haem Fe-binding affinity	Fe^{2+}	Fe^{3+} , Fe^{2+}	[23,117]
Haem association rate constant ($\mu\text{M} \cdot \text{s}^{-1}$)	0.5	22	[23]
Activation of s-GC (fold)	5	400	[117,100]
Vasoactivity	Dilation	Dilation	[23,100]
Smooth muscle effect	Relaxation	Relaxation	[183]
Platelet-aggregation effect	Inhibition	Inhibition	[184,185]
Ischaemic/reperfusion injury	Protection	Protection	[186–189]

**Scheme 1 Illustration showing how NO and CO may interact and compete to affect the generation and effector functions of each other**

The generation of NO occurs via the family of NOS enzymes that convert L-arginine into L-citrulline, resulting in the release of NO. Haem is degraded by the HO family of enzymes, resulting in the liberation of Fe(II), CO and biliverdin. The biliverdin is then converted into bilirubin by the enzyme biliverdin reductase. In terms of the interactions between NO and CO, CO can bind to the haem centre of NOS and inhibit its activity. Moreover, Fe(II) liberated from the degradation of haem can result in a decrease in iNOS expression, which occurs at the level of transcription. On the other hand, NO can induce the expression of HO-1, which subsequently results in CO synthesis. Nitric oxide that is bound to haem centres can be displaced by CO, and the liberated NO can then bind Fe released from the degradation of haem via HO.

carboxylase [31] and caspases [32]. In contrast, NO⁺ reacts with metals to form the NO⁺ ion, which then reacts with thiol groups to form *S*-nitrosothiols (e.g., *S*-nitrosocysteine, *S*-nitroso-glutathione and *S*-nitrosoalbumin).

The interaction of the NO⁺ cation with thiol groups to form *S*-nitrosothiols may be its most important reaction under physiological conditions [22]. In biological systems, *S*-nitrosothiols prolong the half-life of NO, and have been proposed to be the major storage and transport form of NO [13,22,33–35]. For example, *S*-nitrosoalbumin could serve as a source and 'sink' of NO [9,36].

In terms of the chemical interaction between CO and NO, it is known that CO can stimulate NO release from proteins and the production of peroxynitrite [37]. This could be critical in terms of mobilizing intracellular NO and be part of the synergistic interactions of these two molecules. Piantadosi [23] has suggested that CO could cause redistribution of NO in cells, which is consistent with the different equilibrium constants of these molecules for metal binding. For instance, considering Hb, the affinity of NO for Fe(II) is 1500 times greater than CO [21]. However, for sperm-whale (*Physeter macrocephalus*) myoglobin, the overall association rate constants for CO and NO are 0.5 and 22 $\mu\text{M} \cdot \text{s}^{-1}$ respectively [38]. Clearly the presence of Fe will dramatically influence the roles of NO and CO, and, in turn, these signalling molecules will affect cellular Fe metabolism.

Despite NO having a higher affinity for haem Fe, a paradoxical effect of CO is its ability to displace NO after a period of time (Scheme 1). This is because the association constant of NO for haem Fe is greater than that of CO, while the dissociation constant for NO is also greater than that for CO [23]. Thus, as NO is displaced by CO, CO remains bound to haem for a longer period of time [23].

Underlying the similarities between NO and CO are small differences in chemistry that may ultimately cause a large divergence in effector functions. Indeed, the effector functions of CO and NO on the same molecule can be markedly different. A good example is their effect on Hb. It is well known that CO binds to the haem of Hb and prevents oxygen dissociation [39], whereas NO binds not only to haem, but also *S*-nitrosylates the thiol groups [27] that may be involved in regulating respiration [13]. In fact, the ligation of oxygen to Fe(II) in Hb promotes the binding of NO to Cys⁹³ in the β -chain, forming *S*-nitroso-Hb [27]. Deoxygenation of Hb enhances the release of NO, a potent vasorelaxant, which increases blood flow and, consequently, O₂ transport into tissues. Moreover, Hb deoxygenation causes an increase in ventilation via *S*-nitrosothiols that stimulate the ventilatory response to hypoxia [13].

METABOLISM OF Fe AT THE ORGANISM AND CELLULAR LEVEL

Before discussing the roles of NO and CO and their effects on cellular Fe metabolism, we will briefly describe how cells metabolize Fe (for detailed reviews, see [40–42]). Fe is transported in the serum bound to the glycoprotein transferrin (Tf), which binds two atoms of high-spin Fe(III) [43]. A major source of Fe for loading Tf comes from macrophages that recycle Hb Fe [40,44,45]. However, it remains poorly understood how the Fe is released from macrophages of the reticuloendothelial (RE) system, although a putative Fe export molecule, ferroportin1 [also known as Fe-regulated transporter 1 ('IREG1') and metal transport protein 1 ('MTP1')], has recently been described [46–49].

In humans, Fe is not actively excreted and is highly conserved [44]. The physiological losses in men are small, where minute

losses of 1–2 mg daily occur from faeces, urine, secretions, skin, hair and nails. In women, greater losses occur through menstruation and pregnancy [50]. Most body Fe is found in Hb and recycled by cells of the RE system, which scavenge senescent erythrocytes by phagocytosis (Scheme 2) [41,50]. The globin portion of Hb is degraded into amino acids, while haem is broken down by haem oxygenase-1 (HO-1) of the HO family of enzymes [51]. The HO enzymes result in the production of CO, Fe(II) and biliverdin (Scheme 2). Owing to the high affinity of CO for Fe [23], it is suggested that a CO–Fe complex could result from this reaction (see the section below entitled 'The CO–Fe hypothesis of iron metabolism'). In fact, the CO–Fe complex may account for the release of Fe from the erythrocyte-phagocytosing macrophage [52,53], which is subsequently bound by apo-Tf (Scheme 2). Surprisingly, this physiologically critical pathway of Fe mobilization remains largely uncharacterized. Some investigators have suggested that the Fe released from haem via HO is sequestered into ferritin [54]. However, large amounts of Fe are released from macrophages following erythrocyte phagocytosis [52,53], demonstrating that not all Fe is incorporated by ferritin.

Diferric Tf donates its Fe to cells by binding to the specific transferrin receptor 1 (TfR1) on the cell surface (Scheme 2). The Tf–TfR1 complex is internalized via receptor-mediated endocytosis and the Fe released from the protein by a decrease in endosomal pH mediated by a proton pump [43]. The Fe released is then reduced to Fe(II) by an unknown membrane reductase and then transported through the membrane by the natural resistance-associated macrophage protein-2 [Nramp2; also called the divalent metal ion transporter 1 ('DMT1')] (Scheme 2) [41,55–57].

Apart from the TfR1, more recent studies have identified a second Tf-binding protein known as TfR2. TfR2 has a lower affinity for Tf than does TfR1, and its role in Fe uptake and cellular metabolism remains unclear [58,59]. However, it is known that critical mutations or deletions in this gene for this receptor can result in a haemochromatosis [60,61], suggesting that TfR2 plays a role in Fe homeostasis [62]. Apart from the specific high-affinity TfR1, a number of cell types also possess non-receptor-mediated mechanisms of Fe uptake that are consistent with pinocytosis of Tf [63–67].

Once Fe enters the cell it becomes part of a poorly characterized compartment known as the 'intracellular labile Fe pool', the molecular identity of which remains unclear. It has been speculated to be composed of low- M_r ligands such as amino acids and citrate [68]. However, low- M_r intermediates have never been identified during the Fe-uptake process [69,70]. Alternatively, Fe could be bound to high- M_r chaperone molecules similar to those that bind copper [71], or intracellular Fe transport may require the interaction of organelles [70,72,73]. The Fe in this pool is critical, since it is used for the synthesis of haem and [Fe-S]-containing proteins or can be used for Fe storage in ferritin [74,75]. Ferritin is a large polymeric protein composed of 24 subunits of two types: H (heavy) and L (light) [74,75]. Ferritin can store up to 4000 atoms of Fe within its protein shell and may be capable of releasing this metal ion when required [74]. However, the mechanism of ferritin Fe mobilization remains unclear and remains an explorable niche.

The intracellular Fe pool regulates two mRNA-binding molecules known as iron-regulatory proteins 1 and 2 (IRPs) [40,76,77]. Both IRP1 and IRP2 are *trans*-regulators that post-transcriptionally control the expression of a variety of molecules that play essential functions in Fe homeostasis [40,76,77]. The IRPs bind to hairpin-loop structures called iron-responsive elements (IREs). These IREs are found in the 5'- or 3'-

There is some controversy over whether mtNOS is a novel molecule or if it could simply be a membrane-bound iNOS contaminant from other organelles (e.g. lysosomes) in the mitochondrial fraction [25]. However, if it is a novel mitochondrial enzyme, the implications for Fe metabolism are vast, as the mitochondrion is the only site of haem synthesis within the cell [45]. Considering the tremendous rate of Fe uptake by the mitochondrion in erythroid cells [45], in addition to the data that NO binds Fe and can affect intracellular Fe trafficking (see the section entitled 'Iron-containing targets of NO and CO'), it may be worthwhile investigating whether this reaction plays at least some role in the anaemia of chronic disease. Indeed, this condition has been suggested to have some linkage to NO and Fe metabolism [40].

Feedback control of NOS activity is provided by the ability of NO to bind to the haem centre of this molecule and inhibit its activity [89]. A difference in the regulation between the constitutive NOS enzymes and iNOS is that the constitutive proteins are Ca²⁺/calmodulin-dependent, whereas iNOS activity is Ca²⁺/calmodulin-independent [90]. Significantly, the expression of iNOS is affected by the concentration of intracellular Fe [91]. This level of regulation occurs at the transcriptional level when this enzyme is induced using IFN- γ /LPS in macrophages *in vitro* [91]. Increased levels of intracellular Fe decrease iNOS expression and NO generation in these cells, while Fe-deprivation results in enhanced iNOS expression and NO formation [91].

The HO family of enzymes

The production of CO is mediated by the enzyme HO [92], which exists as a macromolecular complex in the endoplasmic reticulum together with cytochrome *c* reductase and biliverdin reductase [93]. There are three HO isoenzymes, namely HO-1, HO-2 and HO-3, of which only HO-1 is inducible [94,95]. Although HO-1 plays an essential role in the degradation of Hb-derived haem, it is also a stress protein known as heat-shock protein-32 ('HSP32') [96]. Generally, HO-1 is thought to play a cytoprotective role against oxidant insults [13,88,97]. However, more recent studies have suggested that the generation of Fe(II) by HO activity can result in pro-oxidant effects under some conditions [98,99].

The expression of HO-1 is ubiquitous in mammalian tissues and can be particularly up-regulated in the spleen and liver [100]. The expression of the molecule commonly increases after exposure to haem derived from senescent erythrocytes or from haemoproteins [29]. Other inducing factors include nutrient depletion [20], hypoxia [101], hyperoxia [102], LPS, phorbol esters, UV radiation, H₂O₂, NO, heavy metals or organic chemicals [4,51,95,103–106].

The HO-2 isoform is expressed in the central nervous system, endothelial cells and interstitial cell networks, and, like HO-1, is thought to be cytoprotective [29,107–109]. The function of HO-3 remains unclear, but it has been cloned from rat brain [110], suggesting a neural function. This enzyme is structurally similar to HO-2, but is less efficient at degrading haem [111]. Apart from the effect of NO at inducing HO-1 [95,106] (Scheme 1), a further intriguing interplay between NO and CO is suggested by the fact that HO-2 can act as a 'sink' for NO [112]. Finally, in addition to CO being synthesized by HO, very small amounts are generated as a by-product of Fe-catalysed lipid peroxidation [113].

Interactions between NOS and HO

There is some interplay between NOS and HO-1 activity (Scheme 1). Both enzymes have a haem centre within the protein, although the haem in HO-1 is transiently bound as a substrate, but still

could be a target for NO [95]. The CO produced from HO can bind to the Fe in the haem centre of NOS, decreasing its activity (Scheme 1) [4,114–116]. Considering that HO can degrade haem, its activity could theoretically inhibit the synthesis of active NOS, which requires this prosthetic group for catalysis [117]. It has been shown that Fe(II), which is a product of HO, also decreases iNOS expression [91] (Scheme 1). The final HO product, biliverdin, is reduced to bilirubin by biliverdin reductase, and this reaction is NADPH-dependent. The synthesis of NO by NOS also requires NADPH. Thus, competition between biliverdin reductase and NOS could potentially reduce the activity of these enzymes by decreasing NADPH availability [117].

It has been shown that NO can increase HO-1 expression and activity at the level of gene expression [11,95,118] (Scheme 1). However, there is some controversy as to the precise molecular mechanism(s) involved. For instance, it is not clear if the effect of NO is cGMP-dependent [118] or -independent [95,119]. Interestingly, NO generated by spermine diazeniumdiolate ('spermine NONOate') increased HO-1 expression by increasing its rate of transcription and by stabilizing HO-1 mRNA [95]. Moreover, the protein-synthesis inhibitor cycloheximide prevented the NO-mediated increase in HO-1 expression, suggesting that protein expression was required for this process [95]. In contrast, later studies by others reported translation-independent stabilization of HO-1 mRNA [120]. The ability of NO to induce HO-1 expression appeared to be related to cellular redox status in some experimental systems [95]. However, other studies did not confirm this, and suggested the role of the mitogen-activated protein kinases 'ERK' (extracellular-signal-related kinase) and p38 [121]. Apart from the above mechanisms, NO may induce HO-1 expression by inducing the loss of haem from haemoproteins [122].

Several papers have described a seemingly contradictory finding, namely that 'NO' decreased HO-1 activity [123,124]. However, on closer examination, these two reports used sodium nitroprusside (SNP) and 3-morpholinosydnonimine hydrochloride (SIN-1) as 'NO donors'. SNP actually releases little NO and has been described as an NO⁺ donor [22,28], while SIN-1 generates mainly ONOO⁻ [28,125]. In addition, it is well known that SNP can release Fe in the presence of cellular reductants as well as a host of other by-products (e.g., cyanide, ferricyanide etc.) [28]. Hence, these latter studies cannot be compared with the work of Hartsfield et al., who used an NO⁻ generator [95].

Both iNOS and HO-1 are induced by some common stimuli, including reactive oxygen species, cytokines and endotoxins [4,126], while sheer stress and cyclic stretch up-regulate HO-1 only [127]. It is noteworthy that HO-2 and NOS are co-localized in blood vessels and the autonomic nervous system [107], suggesting a possible co-ordination of biological roles [128]. In fact, in knockout mice that do not express HO-2, smooth-muscle cells are depolarized, and this is enhanced in double knockout mice, where there was genomic deletion of HO-2 and nNOS [12]. In the HO-2 knockout mice, which express nNOS, the response of jejunal smooth-muscle cells to electrical fields was almost absent, whereas the addition of exogenous CO resulted in restoration of the normal response [12]. These results suggest that, at least in jejunal smooth muscle, the NO system is dependent on the production of CO [12]. If the synergism between NO and CO is more widely applicable, this could have broad physiological implications for diverse biological processes. For instance, concomitant CO production may be necessary to 'drive' NO from stores bound to Fe within the cell (e.g., haemoproteins). As noted previously in the subsection entitled 'The HO family of enzymes', it is noteworthy that HO-3 is found within rat brain [110].

Considering the possibly synergy between HO-2 and NOS in the autonomic nervous system [107], it can be speculated that HO-3 in the brain [110] could act together with nNOS in the central nervous system. Obviously, this will require further investigation in terms of the cellular localization of these molecules, but it could be an interesting avenue of research that could have broad biological significance.

Fe-containing targets of NO and CO

Many of the biological effects of NO and CO can be attributed to the fact that both these molecules avidly bind Fe within the active sites of numerous proteins. The best characterized of these are the haem proteins, particularly Hb and myoglobin. In the Hb tetramer, CO binds to one haem centre, which then results in increased affinity for oxygen by the three other haem groups [39]. This shifts the oxyHb dissociation curve to the left and prevents the molecule from unloading oxygen to tissues [39].

Both NO and CO have been shown to bind to the haem centre of soluble guanylate cyclase (sGC) to increase its activity, resulting in higher levels of cGMP [4,129–131]. However, the efficacy of CO at stimulating sGC activity is far less than that for NO, and does not result in the breakage of the bond between the haem Fe(II) atom and the imidazole side chain of His¹⁰⁵ [131]. In fact, the conformational change in sGC induced by NO results in a 100–400-fold increase in activity, while CO induces a 4–6-fold activation of this enzyme [131]. Interestingly, it has been suggested that CO may function as an inhibitory modulator of the NO–sGC–cGMP signalling mechanism in the brain [132]. Under these conditions, CO may act by becoming bound to sGC before NO or by displacing NO from the enzyme.

NO can also attack [Fe-S] clusters within proteins and change their activity via this mechanism. One of the most important examples in terms of Fe metabolism is the effect of NO on the IRPs. In fact, NO can activate the RNA-binding activity of IRP1, which plays a major role in cellular Fe homeostasis [133–137]. The mechanism by which NO exerts its effects on IRP1 is probably both by depleting intracellular Fe and by interacting with its [Fe-S] cluster [138–140]. The relative roles of NO-mediated intracellular Fe release and direct interaction with the [Fe-S] cluster in activating IRP1–RNA binding activity depend on the source of NO, the redox-related state of NO generated and the amount produced [139]. In contrast with IRP1, NO and ONOO⁻ have been reported to decrease IRP2–RNA binding activity [140–142], perhaps by a mechanism involving NO⁺-mediated degradation [142]. However, while this is a possibility, direct measurement of S-nitrosylation of critical thiol groups of IRP2 needs to be determined. Obviously, CO will not be able to act like NO⁺ if this mechanism is applicable, and it remains unknown whether the [4Fe-4S] cluster could be directly attacked by CO. However, it is possible that CO could affect IRP–RNA binding activity indirectly by binding and depleting the intracellular Fe pool that is generally thought to regulate the protein [76]. Understanding these effects of CO could be significant in assessing its effects on whole-cell Fe metabolism, particularly considering the central role that the IRPs play in Fe homeostasis [76].

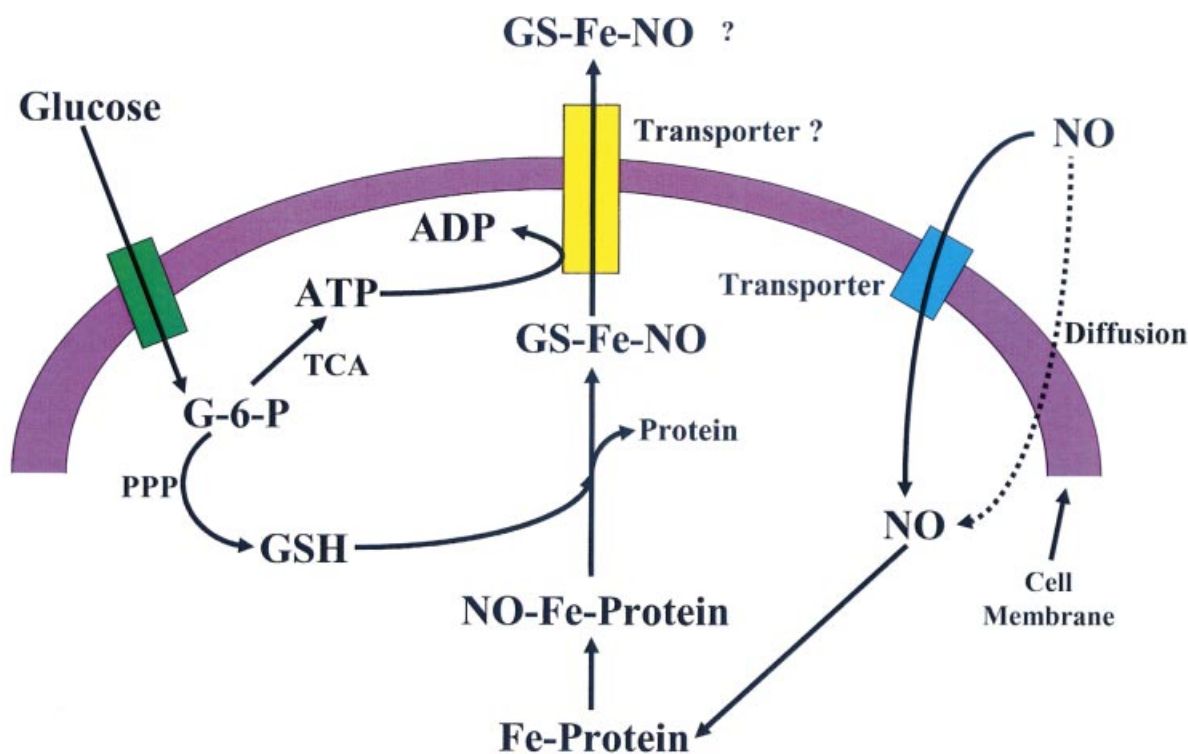
Considering that iNOS can be regulated by intracellular Fe [91], this could lead to an autoregulatory loop whereby low Fe levels induce iNOS. This enzyme subsequently generates NO, which activates IRP1–RNA binding activity [143]. Increased binding of IRP-1 to the 3'-IRE of TfR1 mRNA would increase TfR1 expression, which theoretically leads to elevated Fe uptake from Tf. The enhanced Fe levels in cells would then induce the opposite effect by reducing iNOS transcription, and thus, result-

ing in decreased NO and IRP1–RNA-binding activity [143]. Upon this complex level of control, other studies using murine macrophages have shown that IRP1 gene expression can be down-regulated by NO [144]. Moreover, while NO can induce an increase in TfR1 expression, the increase in Fe uptake from Tf was only minimal [145]. This is probably because NO can also mobilize Fe from cells and inhibit Fe uptake from Tf [139,146].

NO can also form complexes and/or remove Fe from a range of isolated Fe-containing proteins, including ferritin [147,148], ribonucleotide reductase [149], haem-containing proteins [89,150], ferroxidase [122] and lactoferrin [151]. NO can decrease Fe uptake from Tf by cells via interfering with intracellular Fe trafficking and inducing cellular Fe mobilization [136,139,152–154]. Considering that NO forms a complex with Fe in lactoferrin [151], it could be expected that, on the basis of their homology [155], a comparable reaction could occur with Tf. However, the ability of NO to reduce Fe uptake from Tf was not due to direct removal of Fe from the protein, nor to its ability to form an NO–Fe complex within the molecule [145,146]. In fact, the ability of NO to decrease Tf-bound Fe uptake appeared to be mediated by its ability to inhibit ATP production [146], which is essential for cellular Fe internalization [43]. It is questionable whether NO can form an NO–Fe complex with the lactoferrin–Fe complex, as previously reported [151] and described above. This is because of the lack of such an interaction within Tf [146] and the fact that the Fe-binding sites of these molecules are very similar [151].

Regarding the effect of NO on cellular Fe release, initial studies using NO⁺ generators (e.g. SNP) [22] or ONOO⁻ donors (e.g. SIN-1) demonstrated that these agents did not result in appreciable Fe release [136], whereas NO⁻-releasing agents showed high efficacy [139,152]. In experiments using activated macrophages, marked Fe release was observed from tumour target cells [86,87]. This was suggested to be due to the ability of NO to bind and/or remove Fe from proteins (e.g., aconitase) containing [Fe-S] clusters, which play critical roles in energy metabolism [156,157]. Moreover, NO has been shown to inhibit the activity of ribonucleotide reductase [149], which catalyses the rate-limiting step in DNA synthesis. Other Fe-containing targets of NO include NADH:ubiquinone oxidoreductase and succinate:ubiquinone oxidoreductase of the electron-transport chain [158]. Since Fe is crucial for both energy generation and DNA synthesis, the generation of NO by activated macrophages contributes to their cytotoxic surveillance mechanism against tumour cells and pathogens [86,87].

Whether CO binds to any of the above Fe-containing molecules remains largely unknown. However, the fact that NO and CO share similar chemical properties means that this is a possibility that warrants investigation. It may be useful to try and identify CO–Fe complexes either in cells or tissues that express high levels of HO. The existence of NO–Fe complexes in biological organisms has already been documented [158–160], and similar data is required for CO. For example, owing to their high levels of HO-1, erythrocyte-phagocytosing macrophages in culture could be assessed using techniques such as IR spectroscopy that is used by chemists to assess Fe–CO complexes [16]. This type of data is required initially to unequivocally prove the interaction of CO and Fe in cells. Similarly, the organs (e.g. spleen) of animals suffering experimentally induced haemolytic anaemia could be of interest to assess for the existence of CO–Fe complexes. More detailed analysis of Fe-containing proteins in tumour cells, such as ribonucleotide reductase, which exhibit unique EPR signals due to their Fe centres [149], could be assessed using EPR spectroscopy after exposure to activated macrophages or CO-generating agents [15].



Scheme 3 Hypothetical model of D-glucose-dependent NO-mediated Fe mobilization from cells

D-Glucose is transported into cells and is used by the tricarboxylic acid cycle ('TCA') for the production of ATP and by the pentose phosphate pathway (PPP) for the generation of pentose sugars and NADPH. This reductant is involved in the synthesis of GSH. NO either diffuses or is transported into cells where it intercepts and binds Fe bound to proteins or Fe that is *en route* to ferritin. The high affinity of NO for Fe results in the formation of an NO-Fe complex, and GSH may either be involved as a reductant to remove Fe from endogenous ligands or may complete the Fe co-ordination shell along with NO. This complex may then be released from the cell by an active process requiring a transporter. See the text for details. The Scheme is taken from [153] and reproduced with the permission of Blackwell Publishing.

Mechanism of NO-mediated Fe release from cells

Investigations using EPR spectroscopy suggest the formation of Fe-dithiol-dinitrosyl complexes and haem-nitrosyl complexes in activated macrophages and their tumour-cell targets [158–160]. These investigations clearly demonstrate the interaction of NO with Fe in cells. The fact that activated macrophages can release 64% of cellular Fe from tumour-cell targets within a 24 h period [86] illustrates the importance of NO-mediated Fe mobilization and its role in the cytotoxic mechanisms of macrophages.

Recently, the mechanism of NO-mediated release of Fe from cells has been shown to be GSH- and energy-dependent and reliant on the uptake and metabolism of D-glucose [152,153]. Scheme 3 is an illustration summarizing a model of NO-mediated Fe release based upon these investigations [152,153]. Glucose enters the cell by the family of glucose transporters [161] and is subsequently phosphorylated to glucose 6-phosphate [162]. It is then metabolized by two major pathways, through glycolysis and the tricarboxylic acid cycle to form ATP, or through the pentose phosphate pathway to form reduced NADPH (e.g. for GSH synthesis) and pentose sugars [162]. Our studies demonstrated that D-glucose uptake and metabolism by the pentose phosphate pathway, rather than glycolysis, was essential for NO-mediated Fe release [152]. Significantly, depletion of GSH using the specific GSH synthesis inhibitor buthionine sulphoximine [163] prevented NO-mediated Fe release from cells [152]. In addition, this could

be reconstituted by incubation of cells with *N*-acetylcysteine, which increased cellular GSH levels [152].

It is probable that the effect of D-glucose on stimulating NO-mediated Fe mobilization from cells was not just due to its effect on GSH metabolism. Indeed, our experiments have shown that NO-mediated ^{59}Fe release was temperature- and energy-dependent, suggesting that a membrane-transport mechanism could be involved [152]. As shown in Scheme 3, NO can enter cells through diffusion or by a transport molecule such as the protein disulphide-isomerase that catalyses transnitrosylation [164]. A major intracellular target of NO appeared to be the Fe storage molecule ferritin [153]. NO prevented the uptake of Fe into ferritin and also appeared to indirectly mobilize Fe from the protein [153]. An indirect mechanism of Fe release was postulated, since NO-generating agents added to lysates had no effect on ferritin Fe mobilization [153]. The efflux of Fe from cells and its removal from ferritin was GSH-dependent, and could be inhibited using buthionine sulphoximine [152,153]. GSH may assist in the removal of the Fe from the cell by either acting as a reducing agent or by filling the co-ordination shell of an Fe complex composed of NO and GSH ligands. This complex could be lipophilic enough to pass through the cell membrane to exit the cell. However, experimental evidence shows that NO-mediated Fe removal from cells is possibly an energy/ATP-transport-mediated event [152,153]. The transporter involved remains unknown at present, but it could correspond to the Fe

export molecule ferroportin 1 [48] or the ATP-binding cassette ('ABC') family of transporters (e.g. glutathione-S-conjugate export pump), which mediate the export of GSH conjugates [165,166]. Further studies are underway to determine the exact mechanism of transport.

Many investigations examining the effects of NO on cellular Fe metabolism or Fe-containing proteins have utilized a variety of NO-generating agents [e.g. *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosoglutathione (GSNO) etc.] [133,135,138,139,140,152,153]. A valid criticism of these studies could be that these agents are being added to biological systems at high concentrations (e.g., 0.05–0.5 mM) and the quantity of NO produced may not be physiologically relevant. For instance, it could be argued that the Fe release from cells observed after incubation with NO generators such as GSNO or *S*-nitroso-*N*-acetylpenicillamine [139,152,153] does not mirror the biological system. However, it should be noted that activated macrophages added to cells induces NO-mediated Fe release, which inhibits proliferation [86,167]. Further, it is noteworthy that nitrite production by GSNO or *S*-nitroso-*N*-acetylpenicillamine at high concentrations (i.e. 0.5 mM) increases linearly as a function of time and, after 3 h, results in accumulated nitrite levels of 30–40 μM [152]. Nitrite in the serum of normal subjects is approx. 20 μM and this can increase to 250 μM in pregnancy [168]. Since the effect of NO on Fe release and its metabolism can be observed at concentrations of NO generators as low as 0.05 mM [153], it is apparent that these investigations are consistent with physiological conditions. Finally, it should be mentioned that the use of NO generators with appropriate controls (e.g. for GSNO, the control is GSH [139,152,153]) provides an experimental tool that allows understanding of the interactions of NO and Fe within cells. Such an approach should also be complemented with studies of the interaction of NO-producing activated macrophages with tumour-cell Fe metabolism.

Could CO act as a cytotoxic effector molecule that acts synergistically with NO?

Recent studies have suggested that CO generated by HO-1 can act as an autocrine inhibitor of vascular smooth-muscle growth [169]. Further, some growth factors act to increase HO-1 expression, and it has been suggested that the increase in CO production may act to modulate or fine-tune proliferation [106,170,171]. Since NO is thought to induce HO-1 expression [95,119], it can be speculated that marked NO production in activated macrophages could increase HO-1 expression and CO synthesis. Theoretically, CO may work synergistically with NO to act as a cytotoxic effector. The production of small quantities of CO in the macrophage could induce greater NO release from these cells by mobilizing intracellular NO pools (e.g., from haemoproteins), as seen under other conditions [37,172]. Considering the role of CO in immune function, Sato et al. [53] have shown that the CO generated by HO can prevent rejection of mouse to rat cardiac transplants, suggesting modulation of the cytotoxic effector response. Obviously, further studies are required to examine the role of CO in immunological function and cell-mediated cytotoxicity.

The CO–Fe hypothesis of Fe mobilization

As described previously, the HO family of enzymes produces CO through the breakdown of haem, liberating CO, biliverdin and Fe in stoichiometric amounts [92]. Owing to the high affinity of CO for Fe [23], a CO–Fe complex could result. In fact, *in vitro*, the formation of an Fe(II) complex with cysteine and CO ligands have been reported [173–175]. It is tempting to speculate that

CO–Fe–thiol complexes may then diffuse from the macrophage. The Fe from the unstable Fe–thiol complexes should readily bind to Tf, and the CO be trapped by Hb [23]. Hence the CO–Fe complex may account for the marked release of Fe from erythrocyte-phagocytosing macrophages [41].

Apart from the known ability of NO to form intracellular Fe complexes and release Fe from cells [86,139,146,152–154], and the possible role of CO in this process [20], numerous observations are consistent with the CO–Fe release hypothesis. For instance, following erythrocyte phagocytosis, about 70% of Fe is rapidly released in a labile form from macrophages ($t_{1/2} \approx 40$ min) [44,53,176]. Furthermore, phlebotomy, by causing tissue hypoxia and increased erythropoietin release, stimulates erythropoiesis by 2–8-fold [177]. The extent of stimulation, which depends on the source of Fe, is by far the highest if Fe is provided by Hb catabolism [177]. We are not proposing that Fe is exclusively released via 'CO–Fe'. Indeed, apo-Tf and the ferroxidase caeruloplasmin may facilitate Fe release [178,179].

Detailed studies are required to test the CO–Fe hypothesis. Intriguingly, a previous investigation by Ferris and colleagues [20] showed that cells transfected with HO-1 resulted in a decrease in Fe uptake from Tf and an increase in Fe mobilization from cells. These observations are very similar to the effects observed with NO on decreasing Fe uptake from Tf and increasing Fe efflux from cells [139,145,152,153,167]. However, it was not clear whether these effects were mediated by CO or other products of haem degradation [20]. Further experiments using pure CO or CO-generating compounds [15] are essential to further test this hypothesis.

Additional support for the CO–Fe hypothesis comes from studies using HO-1 and HO-2 knockout mice [180,181] and from a child deficient in HO-1 [182]. Studies using the HO-1 knockout mouse demonstrated that this enzyme was required for Fe re-utilization from Hb as well as facilitating Fe release from hepatic and renal tissue [180]. The adult mice developed anaemia associated with low serum Fe, but showed high levels of Fe in the liver and kidneys [180]. Similarly, Fe deposition in the liver and kidneys was found in a child deficient in HO-1 [182]. In contrast, in HO-2 knockout mice there was Fe accumulation in the lungs [181].

Collectively, the role of HO-1 in the re-utilization of haem Fe is critical, and there is evidence that CO–Fe complex could be the form of Fe released from macrophages after erythrocyte phagocytosis. This hypothesis is clearly testable and could provide new insights into the regulation of intracellular Fe metabolism.

SUMMARY AND CONCLUSIONS

Both NO and CO share a high affinity for Fe, which results in their ability to act as effectors for a range of biological processes. The interaction with Fe-containing targets suggests that, depending upon the conditions, CO and NO may act synergistically or in competition in terms of their biological effects. The effect of NO on cellular Fe metabolism has been well characterized over the last 10 years. In contrast, studies on the effect of CO on Fe metabolism are in their infancy. However, from preliminary investigations, several intriguing glimpses of the rich biological chemistry of CO and Fe have been uncovered. These include the possible function of CO in both preventing Fe uptake from Tf and increasing Fe mobilisation from cells. The CO–Fe complex could also play an important role in the physiological release of Fe from erythrocyte-phagocytosing macrophages ('the CO–Fe release hypothesis'). In addition, synergy between CO and NO may theoretically be important in the cytotoxic effector mechanism of activated macrophages.

Considering the likely effects of CO on cellular Fe pools, its competition with NO for Fe, and the interactions between HO and NOS, it remains to be explored what effect CO will have on the regulation of cellular Fe metabolism. In particular, it will be of interest to determine the effects of CO on IRP–RNA binding activity. Clearly, if a CO–Fe complex can form, this will result in the disturbance of intracellular Fe pools that could affect IRP–RNA binding activity and a variety of vital metabolic pathways. In turn, the effect of CO on Fe may subsequently influence iNOS expression, which is regulated by this metal ion.

In conclusion, the interaction of NO and Fe is important to understand, as it plays a critical role in a variety of metabolic pathways that include the regulation of blood pressure and the killing of tumour cells by activated macrophages. Similarly, the high affinity of CO for Fe has been known for centuries [17]. However, the possibility that it could play synergistic or antagonistic roles with NO in terms of its interaction with Fe is a fascinating area of research that, to date, has only been superficially explored.

D.R.R. thanks the National Health and Medical Research Council and the Australian Research Council for grant and fellowship support. We kindly thank Juliana Kwok, Sarah Tandy and Nghia Le for their suggestions on the manuscript prior to its submission.

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Received 19 August 2002/5 November 2002; accepted 7 November 2002

Published as BJ Immediate Publication 7 November 2002, DOI 10.1042/BJ20021302