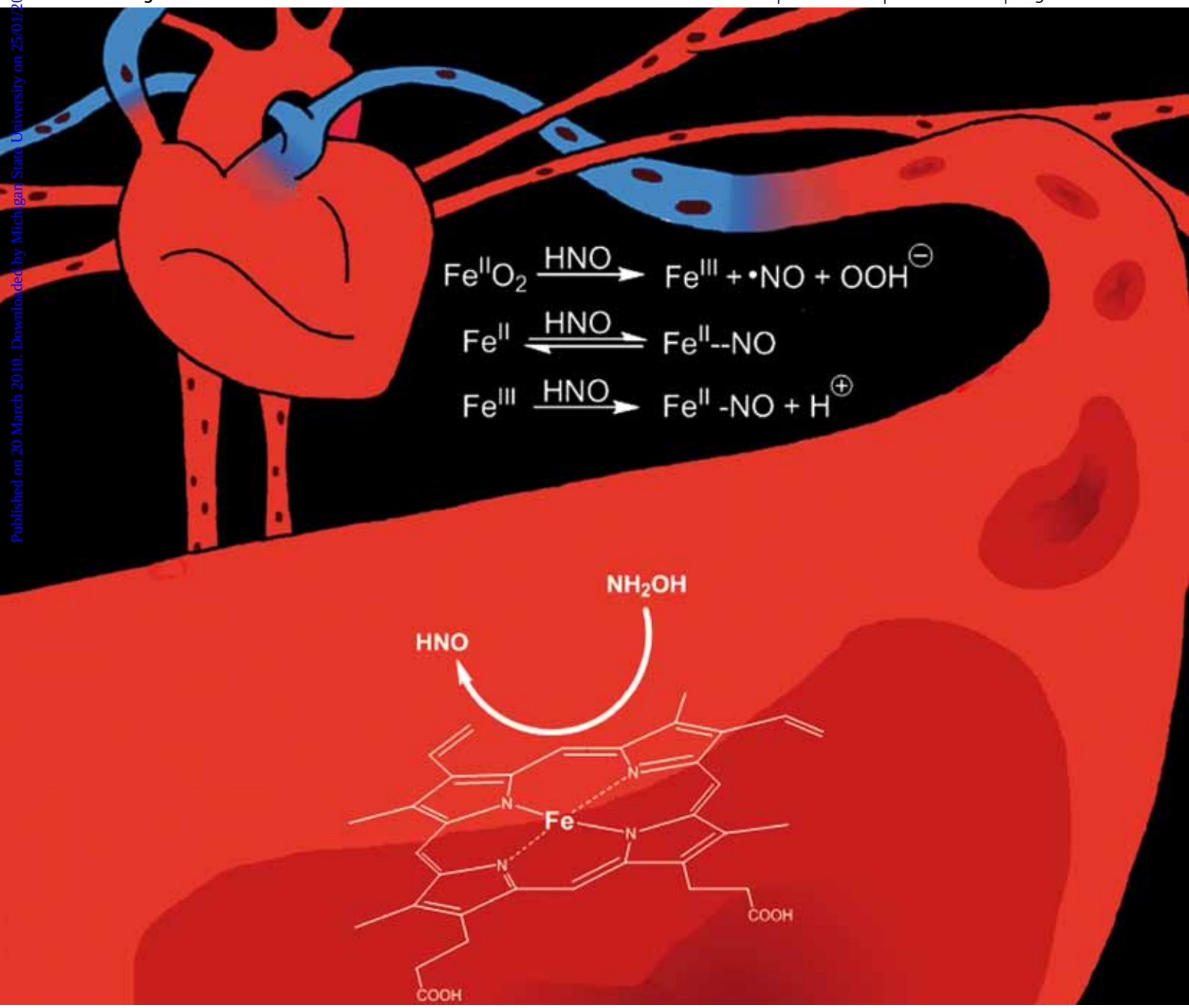
Dalton Transactions

An international journal of inorganic chemistry

www.rsc.org/dalton

Volume 39 | Number 22 | 14 June 2010 | Pages 5189–5400



ISSN 1477-9226

RSC Publishing

1477-9226(2010)39:22;1-7

Oxidative heme protein-mediated nitroxyl (HNO) generation

Julie A. Reisz, Erika Bechtold and S. Bruce King*

Received 15th January 2010, Accepted 25th February 2010
First published as an Advance Article on the web 20th March 2010
DOI: 10.1039/c000980f

The distinct biological properties of nitroxyl (HNO) have focused research regarding the chemistry and biology of this redox relative of nitric oxide (NO). Much of HNO's biological activity appears to arise through modification of thiol-containing enzymes and proteins and reactions with iron-heme proteins. The reactions of HNO with hemoglobin and myoglobin serve as a general model for understanding HNO reactivity with other heme proteins. Interaction of HNO with catalase and soluble guanylate cyclase may have biological roles. While endogenous HNO formation remains to be described, we summarize work that reveals HNO formation through oxidative heme protein metabolism of various nitrogen-containing substrates including hydroxylamine, hydroxyurea, hydroxamic acids, cyanamide, and sodium azide. Depending on the enzyme, the nascent HNO reductively nitrosylates the heme protein or escapes the heme pocket as HNO. Such results define an alternative metabolism-based route to HNO that may inform endogenous HNO production.

1. Nitroxyl

The establishment of nitric oxide (NO) as an important mediator in the vascular, neural, and immune systems has brought attention to other nitrogen oxides as NO sources or independent biological signaling agents.[1] Studies from numerous laboratories over the last 20 years show that nitroxyl (HNO), a structurally similar compound related to NO by one-electron reduction and protonation, elicits biological and pharmacological activities distinct from NO. These findings have driven work to better understand the chemistry and reactivity of HNO, to discover alternative methods of HNO generation and detection, and to develop HNO as a therapeutic entity. A number of excellent recent reviews detail HNO chemistry, biochemistry, biology, and therapeutic potential.[2-5] This perspective will briefly review HNO biology and chemistry, summarize the known reactions of HNO with heme proteins, and examine HNO formation by heme protein-mediated oxidation of various substrates.

Department of Chemistry, Wake Forest University, Winston-Salem, NC, 27109, USA. E-mail: kingsb@wfu.edu; Fax: 336-758-4656; Tel: 336-758-5774

1.1 Nitroxyl biology

Nitroxyl (HNO) releasing compounds consistently display biological actions that often differ from NO or NO donors and these activities have been reviewed in detail.[2,3] As nitroxyl rapidly dimerizes to hyponitrous acid, which further dehydrates to nitrous oxide, biological studies of HNO rely upon donor compounds.[5-8] Angeli's salt (AS, $Na_2N_2O_3$) spontaneously decomposes at neutral pH to HNO and nitrite and represents the most common HNO source for most chemical and biological studies.[5,9] This requirement of HNO donors highlights the importance of the development of alternative HNO sources.

Early work shows HNO donors inhibit aldehyde dehydrogenase and this activity forms the basis for the use of cyanamide as

Julie A. Reisz

The author received a BS in Chemistry from Allegheny College in 2007. She is currently a third year Ph. D. student in Chemistry at Wake Forest University working under the direction of S. Bruce King. Her current research involves phosphine-mediated reactions of HNO in effort to identify and develop new methods to detect and quantify HNO in biological systems.

Erika Bechtold

The author received a BS degree in Chemistry from Virginia Polytechnic Institute and State University in 2006. She is now pursuing a PhD in Chemistry at Wake Forest University under the direction of S. Bruce King. Her current research includes using novel synthetic approaches to create protein labels for the trapping and detection of cysteine-based post-translational modifications in cells, specifically sulfenic acids and S-nitrosothiols. The author plans on pursuing postdoctoral work in the field of biological chemistry after graduation.

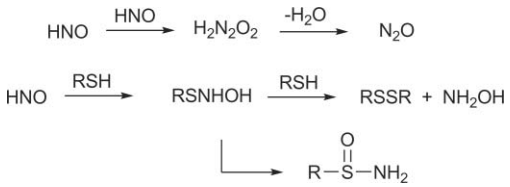
a treatment for alcoholism.[10,11] Nitroxyl releasing compounds exhibit numerous cardiovascular effects including vasorelaxation, an action similar to NO releasing compounds.[12] Angeli's salt enhances myocardial contractility by increasing calcium cycling and by sensitizing myocardial responsiveness to calcium, leading to the suggestion of HNO as a new therapy for congestive heart failure.[13-16] Nitroxyl donors demonstrate a different pattern of protection/exacerbation during myocardial ischemia/reperfusion injury compared to NO donors, further distinguishing the biological activities of these redox-related molecules.[17] HNO reacts with hemoglobin to decrease the amount of NO scavenging by plasma hemoglobin suggesting HNO as a possible therapy for various hemolytic conditions.[18]

Nitroxyl blocks glyceraldehyde 3-phosphate dehydrogenase and this activity forms the basis of potential HNO-based cancer therapies.[19-21] In the presence of oxygen, nitroxyl donors damage DNA in a pattern different from NO donors and Angeli's salt generates hydroxyl radicals under some conditions.[22,23] HNO affects the nervous system by modulating the NMDA receptor, and Angeli's salt enhances ischemic cerebral injury and oxidative neurotoxicity.[24,25] In general, the molecular mechanistic basis of these wide ranging activities appears to arise from HNO's interaction with biologically relevant 1) thiol-containing proteins and 2) metalloproteins. Such reactions yield structural modifications that produce changes in protein function or activity and other nitrogen oxides including NO.

1.2 Nitroxyl chemistry

Miranda comprehensively reviewed HNO chemistry and reactivity in 2005.[5] Various experimental and theoretical studies indicate that nitroxyl predominantly exists in its protonated form (HNO) in aqueous solution at physiological pH with an approximate pK_a of 11.4.[26-28] The nitroxyl anion ($^-$NO) possesses a triplet ground state, similar to oxygen, kinetically retarding HNO deprotonation.[27-29] The relatively high pK_a coupled with the thermodynamic unfavorability of NO to $^-$NO reduction permits HNO to exist as a defined species in biological systems, especially in the absence of oxidants.

Similar to *C*-nitroso compounds and lower molecular weight aldehydes (formaldehyde), nitroxyl dimerizes to hyponitrous acid that dehydrates to nitrous oxide (Scheme 1).[6] Theoretical studies predict favorable thermodynamic parameters for the reaction of HNO with softer nitrogen and sulfur (compared to oxygen) nucleophiles.[27] Experimentally, HNO acts as an electrophile and readily reacts with amines, thiols, and phosphines to generate diazenes, disulfides or sulfinamides, and aza-ylides, respectively.[30-32] Scheme 1 depicts the formation of the products during the reaction of HNO with thiols through the intermediacy of an *N*-hydroxysulfenamide that reacts further to give disulfide or rearranges to sulfinamide. Such reactivity likely mediates a large portion of HNO's thiol-dependent biological activity.

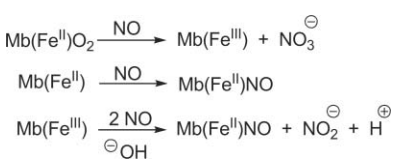


Scheme 1 Electrophilic reactions of HNO.

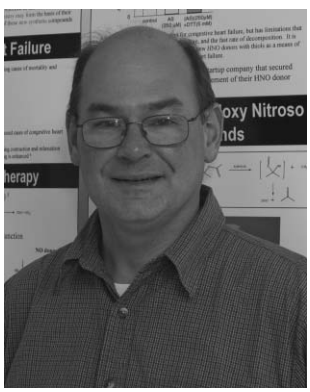
The weak N–H bond strength of HNO allows it to act as an H-atom donor and reducing agent (forming NO) and HNO quenches nitroxide radicals such as TEMPO and polyunsaturated fatty acid radicals.[33-35] Nitroxyl directly reacts with molecular oxygen although the kinetics, products, and mechanism of this reaction remain to be fully described.[36] Electron lone pairs on both nitrogen and oxygen allow HNO to interact with various Lewis acids, particularly metals. Similar to NO, HNO exhibits diverse chemistry with various metals and metal-containing proteins and the next section of this perspective summarizes the reactions of HNO with metal heme-containing proteins.

2. Reactions with heme proteins

Reactions of NO and ferrous and ferric myoglobin (Mb) and hemoglobin (Hb) are well-characterized, play numerous physiological roles, and require consideration before discussing HNO-heme protein reactions. Oxygenated ferrous Mb and Hb serve as rapid and efficient NO traps yielding the oxidized ferric (met) form and nitrate with a rate constant of 5-8 × 10^7 M^{-1} s^{-1} for MbO_2 and 9 × 10^7 M^{-1} s^{-1} for HbO_2 (Scheme 2).[37,38] Deoxygenated Hb or Mb reacts with NO producing ferrous nitrosylated heme at a nearly identical rate (3 × 10^7 M^{-1} s^{-1} for Hb, Scheme 2).[39] Ferric Hb or Mb in the presence of two NO equivalents undergoes reductive nitrosylation forming the ferrous nitrosyl heme and nitrite with an associated pseudo-first order rate constant ranging from 10^4 - 10^7 M^{-1} s^{-1} for metMb.[40-42] Determining the rate for the reaction of



Scheme 2 Reactions of hemoglobin and myoglobin with NO.



S. Bruce King

The author earned BS and MS degrees in forestry and pharmaceutical science from West Virginia University in 1985 and 1988, respectively. He received his PhD in organic chemistry from Cornell University in 1993 under the direction of Bruce Ganem. Following a postdoctoral fellowship with K. Barry Sharpless at the Scripps Research Institute, he joined the faculty of chemistry of Wake Forest University where he is now a professor. The author's main research interests include the development and chemistry of new nitric oxide and nitroxyl donors, the reactions of nitric oxide and nitroxyl with biological targets and synthetic organic chemistry.

metHb with NO is complicated by heme cooperativity, resulting in a slow phase ($k = 5 \times 10^3$ M^{-1} s^{-1}) and a fast phase ($k = 1.4 \times 10^4$ M^{-1} s^{-1}) which give different observed rates.[41] (Scheme 2).

Although the reactions between HNO and heme proteins remain less studied than the reactions between NO and heme proteins, the reactions/interaction of HNO with hemoglobin and myoglobin provide well-characterized examples for comparison.

2.1 Oxyhemoglobin & oxymyoglobin

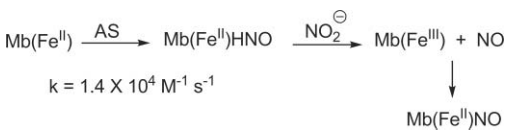
The oxygenated ferrous hemes of myoglobin and hemoglobin undergo rapid oxidation to the ferric forms upon reaction with HNO generated from Angeli's salt (AS). Studies with MbO_2 suggested the reaction to be first order in AS and zero order in heme protein.[43] In addition to ferric heme formation, this reaction also yields nitrate possibly *via* H-atom abstraction of HNO to NO with the heme-bound oxygen being converted to HO_2^- (Scheme 3).[31] Subsequent reaction of nascent NO with oxygenated heme provides additional ferric heme and NO_3^-.[31] Despite this original proposal for nitrate formation,[31] neither NO or HO_2^- generation have been substantiated during this reaction and other mechanisms including the hydroxylamine radical also explain the observed products.[5] Kinetic studies support this mechanism with a 2 : 1 (heme : HNO) stoichiometry and illustrate a role for NO (Scheme 3).[43]

Scheme 3 Reaction of oxyHb or Mb with HNO.

The reaction of HNO with MbO_2 is among the most rapid of the known traps for HNO with a derived rate constant of 1×10^7 M^{-1} s^{-1}.[35] MbO_2 readily oxidizes HNO to NO, which may have important biological implications for endogenous NO generation from HNO. The indistinguishable reactivity of HNO and NO with MbO_2 prevents the use of metMb formation as a marker for HNO release by donor compounds or as an indirect method of *in vivo* HNO quantification.

2.2 Deoxyhemoglobin & deoxymyoglobin

Deoxygenated ferrous hemes directly react with HNO to form coordination complexes and Farmer has recently reviewed HNO as a heme ligand (Scheme 4).[44] Early studies showed the initial reaction of deoxyHb and AS provides HbNO and metHb in equivalent amounts, which was shown to be a two-step mechanism with initial metHb formation followed by HNO-promoted metHb reductive nitrosylation to HbNO in the second.[43,45] The overall reaction mechanism appeared to be second order in AS and zero order in deoxy heme.[45] Ultimately, Doyle suggested that the reaction of deoxyMb with AS produces an intermediate



Scheme 4 Reaction of deoxyHb or Mb with HNO.

ferrous Mb(HNO) complex, which reacts with NO_2^-, giving rise to metMb and MbNO as observed.[31] The reactivity and the spectral similarity of this HNO complex to MbNO likely prevented its detection and complicated the interpretation of these initial reactions.[46]

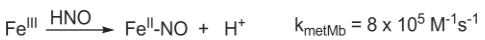
The complexation of HNO with ferrous deoxyhemes remained largely under-appreciated until Farmer demonstrated the reduction of MbNO using Cr^{II} reagents in aqueous alkaline solution provides a ferrous Mb(HNO) complex.[46] Remarkably, the HNO adduct of deoxyMb is stable in anaerobic solution with a half-life of several months making deoxyHb trapping of HNO essentially irreversible. Electrochemical methods oxidize the ferrous Mb(HNO) adduct to MbNO over several minutes.[46]

Purified aqueous samples of ferrous Mb(HNO) are stable over pH 6–10, and as a result, the heme pocket of the Mb(HNO) complex is well-characterized by NMR and molecular modeling.[47] ^{2}D-NMR studies of the ferrous Mb(HNO) complex reveal a hydrogen bond between the HNO proton (15 ppm) and the distal pocket histidine residue (His64) analogous to the hydrogen bonding in MbO_2 known to confer significant stability to oxygen-bound heme complexes.[47] The rate constant for ferrous Mb(HNO) complex formation is estimated at 1.4×10^4 M^{-1} s^{-1}.[48] The ferrous Mb(HNO) complex reacts with AS-derived NO_2^- to provide metMb and NO, which subsequently form MbNO. (Scheme 4).

Exposure of the ferrous Mb(HNO) complex to air, NO_2^-, or NO leads to oxidation to metMb over several minutes.[48] Most recently, Farmer has synthesized stable ferrous Hb(HNO) adducts of human, soy, and clam hemoglobins that may be utilized as heme pocket models for probing oxygen binding parameters. In contrast to oxyhemes, HNO-bound hemes offer characteristic NMR signals (for ^{1}H and ^{15}N) distinct for each heme, a valuable tool for examining mixtures to distinguish proteins and subunits.[49]

2.3 Methemoglobin & metmyoglobin

The heme of methemoglobin (metHb) and metmyoglobin (metMb) is a paramagnetic Fe^{III} 5-coordinate high spin complex.[50,51] In the presence of AS-derived HNO, the ferric heme undergoes reductive nitrosylation, producing a ferrous-nitrosyl complex ($Fe^{II}NO$, Scheme 5).[5,31,52,53] Mechanistic studies suggest a direct complexation between HNO and Fe^{III} followed by electron transfer as opposed to an outer-sphere electron transfer reaction.[54]

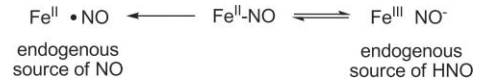


Scheme 5 Reaction of metMb with HNO.

Reductive nitrosylation by AS proceeds with a rate constant of 8×10^5 M^{-1} s^{-1} for metMb and is zero order in metMb and first order in AS.[31,35] With an estimated first order rate constant of 6.6×10^{-4} M^{-1} s^{-1}, AS decomposition to HNO and NO_2^- is significantly slower than the rate of formation of $Mb(Fe^{II})NO$.[53] Determining the rate of reductive nitrosylation of metHb is complicated by the reaction of HNO with the β-93 cysteines of Hb.[31] Monitoring the reaction progress by measuring HbNO formation showed an initial "slow phase" followed by rapid conversion to the nitrosyl.[31] The initial phase is still first order in AS and zero order in metHb.[31] Doyle demonstrated that the chemical modification of the β-93 sulfhydryl groups to the thioacetamide derivative completely

eliminated the initial slow phase for HbNO formation and the observed rate was consistent to that of metMb with AS.[31]

Several ideas exist concerning the possible biological implications of Hb or Mb(Fe^{II})NO complexes. Once formed, HbNO exists in resonance with $Fe^{III}NO^-$ and while not currently substantiated has been proposed as a possible endogenous source of HNO (Scheme 6).[55,56] $Fe^{II}NO$ could also act as a source of NO, however the dissociation of nitric oxide from $Fe^{II}NO$ is very slow with 10^{-3} s^{-1} reported as the fastest rate constant.[57,58] Under physiological conditions, the release of NO from $Fe^{II}NO$ may first require oxidation to $Fe^{III}NO$ as this species has a rate constant for NO release that is about 1000 times higher.[41]

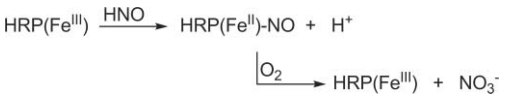


Scheme 6 Iron-nitrosyl complexes as HNO or NO sources.

Five-coordinate ferric heme proteins such as metHb or metMb have been proposed as efficient HNO traps for HNO. The ferrous nitrosyl products have unique UV/Vis absorption and EPR spectra that can be monitored as an indirect measure of [HNO] in samples.[45] This method for detecting HNO remains impractical *in vivo* due to the fact that ferric hemes also react with NO to give the identical Fe^{II}-nitrosyl complex, making the distinction between NO and HNO problematic.[58-60]

2.4 Horseradish peroxidase

The resting state of the peroxidase heme, including horseradish peroxidase (HRP), is a 5-coordinate high spin ferric complex with a proximal histidine ligand.[61] The HRP catalytic cycle commences with binding of H_2O_2 to the ferric heme, oxidation to a ferryl (Fe^{4+}) intermediate (Compound I), and subsequent two-electron substrate oxidation (through a second ferryl intermediate, Compound II) with overall reduction of H_2O_2 to water.[61] As expected for a pentacoordinate ferric heme protein like metHb, HRP forms a ferrous nitrosyl complex upon HNO exposure (Scheme 7).[35,62,63] An estimated rate constant of 2×10^6 M^{-1} s^{-1} was determined for HRP(Fe^{II})NO formation.[35] The HRP(Fe^{II})NO complex undergoes auto-oxidation in the presence of O_2 to give nitrate and regenerate ferric heme (Scheme 7).[60,63,64]

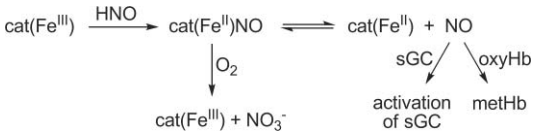


Scheme 7 Reaction of HRP with HNO.

In addition to their antioxidant and protective effects, peroxidases catalyze the conversion of hydroxylamine and hydroxyurea (NH_2OH and $NH_2CONHOH$) to HNO, suggesting possible endogenous sources of HNO that will be discussed in Section 3.[62,63]

2.4 Catalase

Catalase (cat) catalyzes the conversion of H_2O_2 to H_2O and O_2 in a dismutation reaction.[65,66] Cat is also a 5-coordinate ferric heme protein that reacts with HNO to form a ferrous nitrosyl complex with an estimated rate constant of 3×10^5 M^{-1} s^{-1} (Scheme 8).[35,64,67]



Scheme 8 Reaction of cat with HNO.

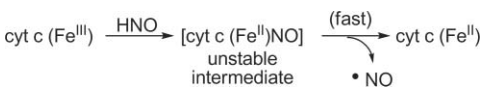
Similar to other ferrous nitrosyl complexes, this catalase complex demonstrates excellent stability under anaerobic conditions, but exposure of cat(Fe^{II})-NO to O_2 results in regeneration of the ferric heme and nitrate (Scheme 8).[67] Ferric catalase resists reduction (sodium dithionite does not reduce ferric catalase) making examples of ferrous catalase complexes rare and only prepared through photochemical reduction sequences.[42,68,69] HNO acts as an effective reductant for ferric catalase and yields cat(Fe^{II})NO making AS the reagent of choice for preparation.[67] Unlike HNO, nitric oxide does not reductively nitrosylate catalase and this reactivity difference between HNO and NO with ferric catalase presents an opportunity to differentiate these redox species.

The preference of the catalase heme iron to remain in the ferric state suggests the possibility of weak ligand binding to the ferrous metal identifying cat(Fe^{II})NO as a possible NO donor. This ferrous nitrosyl complex oxidizes oxyHb presumably through NO release (Scheme 8).[67] Catalase mediated oxidation of hydroxylamine also generates cat(Fe^{II})NO that activates soluble guanylate cyclase (sGC) presumably through NO exchange (Scheme 8).[70,71] The ability of the cat(Fe^{II})NO complex to act as an NO donor may signify an important role for catalase in HNO physiology. Catalase also catalyzes the formation of HNO from hydroxyurea, cyanamide, and sodium azide (NaN_3), and these transformations will be addressed in Section 3.[72-74]

2.5 Cytochrome *c* oxidase

Cytochrome *c* oxidase (cyt c) is a membrane-bound copper-heme enzyme that catalyzes the reduction of O_2 to H_2O in mitochondria during electron transport.[75] The heme pocket (which binds O_2 during normal catalysis) is a high spin 5-coordinate ferric center similar to metMb.[75] NO regulates cyt *c* activity by binding and coordinating at the heme center, inhibiting mitochondrial respiration.[76]

The reaction of HNO with cyt c(Fe^{III}) gives cyt c(Fe^{II}) and NO with an estimated rate constant of 4×10^5 M^{-1} s^{-1} (Scheme 9).[35,77] This reaction most likely proceeds through the direct formation of the iron nitrosyl complex that rapidly dissociates to Fe^{II} and NO, but this reaction may occur through an outer-sphere electron transfer process.[31,35,77,78] Kinetic evaluation of a non-homologous *E. coli* cytochrome *d* oxidase indicates the reaction is first order in HNO and cyt *d* and involves direct interaction between HNO and the heme.[79]



Scheme 9 Reaction of cyt c with HNO.

The addition of AS to purified mitrochondria in the oxidized (non-respiring) state resulted in the measurable formation of NO.[76] However, when AS was incubated with SMPs (submitochondrial particles) devoid of cyt *c*, a similar NO detection profile was

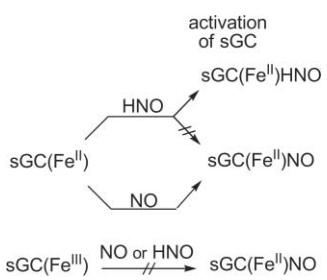
observed, indicating that an additional component in the inner membrane of mitrochondria may also be responsible for the oxidation of HNO.[76]

2.6 Soluble guanylate cyclase

Vasorelaxation, one of the most prominent physiological roles of NO, occurs through activation of the heme protein soluble guanylate cyclase (sGC), which catalyzes the conversion of GTP to cGMP.[80] The vasodilatory mechanism involves binding of NO to the sGC ferrous heme providing a ferrous nitrosyl complex with several hundred fold heightened activity relative to un-nitrosylated protein.[81,82]

Angeli's salt elicits vasodilation of rabbit thoracic aorta and bovine intrapulmonary artery at concentrations 100 times lower than that of nitrite-mediated vasodilation.[12] As nitrite is also an AS decomposition product, these results reveal the potency of HNO.[12] Levels of cGMP were markedly increased following exposure to AS, suggesting HNO-mediated activation of sGC.[12] Additionally, relaxation of rat vasculature by AS is attenuated by sGC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ).[83] Concentration-dependent vasorelaxation of rat mesenteric arteries by AS occurs in the presence of NO-scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) indicating that these effects do not result from HNO oxidation to NO.[84] Overall, these results suggest that HNO itself elicits vasodilatory effects through an sGC-mediated pathway.

A recent study of bovine lung sGC activation by AS and the structurally distinct HNO-donor 1-nitrosocyclohexyl trifluoroacetate (NCTFA) validates direct enzyme activation by HNO (without HNO conversion to NO). In this work, AS and NCTFA enhance the activity of sGC 20- and 60-fold, respectively.[85] Removal of the heme retains basal enzyme activity but significantly diminishes activation by HNO donors, implying that the mechanism of HNO activation involves direct heme binding.[85] Ferricyanide oxidation provided ferric heme sGC that proved inactive toward both NO and HNO donors.[85] Reduction of the enzyme then restored sensitivity to activation by both NO and HNO donors. Given that HNO is not expected to form sGC(Fe^{II})NO from the reaction with ferrous sGC, the prevailing proposed activation mechanism involves direct interaction of HNO and the ferrous heme to form an HNO ferrous heme complex (Scheme 10).[85] Interestingly, inhibition of sGC occurs at higher concentrations of AS (100 μM) likely *via* reaction of excess HNO with protein thiols.[85] Thus, HNO may provide a concentration- and heme-dependent regulation of sGC activity.



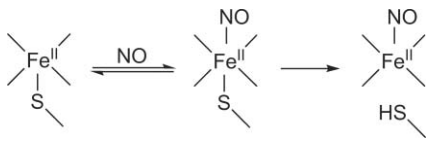
Scheme 10 Possible activation of sGC by HNO.

Trapping of HNO by the ferrous sGC heme may provide an sGC(Fe^{II})HNO adduct similar to complexes for hemoglobin and myoglobin, with activity comparable to the sGC(Fe^{II})NO heme (Scheme 10). Another explanation for HNO-mediated sGC activation would be the formation of active sGC(Fe^{II})NO from the reaction of ferrous sGC and HNO by an unknown mechanism or *in vivo* oxidation of the Fe^{II}HNO. A subsequent report challenges these conclusions, asserting that HNO activation of sGC first involves oxidation to NO, possibly by superoxide dismutase.[86] Certainly the question of sGC activation by HNO donors requires examination as HNO offers promise as a potent vasorelaxant. Rat aortic tissue does not build tolerance towards AS, an impediment to long-term therapy with other nitrovasodilators.[87]

2.7 Cytochromes P450

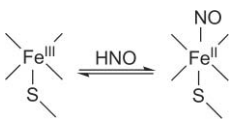
Cytochromes P450 (CYP450) represent a diverse group of heme isoenzymes that serve as ubiquitous oxidizing agents. In mammalian systems, CYP450 participate in the biosynthesis of regulatory compounds such as steroids, prostaglandins, and fatty acid derivatives and the metabolism of drugs and other xenobiotics.[88,89]

In the resting state, CYP450 exists as a pentacoordinate ferric heme with a catalytic cycle that includes ferrous, ferryl, and oxygen-bound complexes. The NO donors DEA/NO ([Et_2NN(O)NO]Na) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) potently inhibit CYP450 *via* several distinct mechanisms.[90] Nitrosylation of the deoxygenated ferrous heme impedes binding of molecular oxygen *via* reversible inhibition.[90] Binding of NO to the heme may also displace the cysteine ligand, allowing oxidation or nitrosation of the cysteine thiol, irreversibly inhibiting the catalytic cycle (Scheme 11).[90,91]



Scheme 11 Inhibition of CYP450 by NO.

Treatment of S9 microsomes with AS increased inhibition of CYP450 comparable to results obtained with SNAP.[52] Removal of the HNO donor regenerates the active enzyme suggesting that both NO and HNO reversibly regulate CYP450 activity.[52] The mechanism of HNO inhibition involves a reductive nitrosylation of the ferric heme resting state, providing the expected ferrous nitrosyl complex formed when NO binds to the deoxy ferrous heme (Scheme 12).[52]



Scheme 12 Inhibition of CYP450 by HNO.

3. Oxidative heme protein-mediated HNO formation

Despite the recent interest in HNO biology, chemistry, and potential therapeutic use, endogenous HNO production eludes conclusive description.[2,3] The biochemical conversion of L-arginine

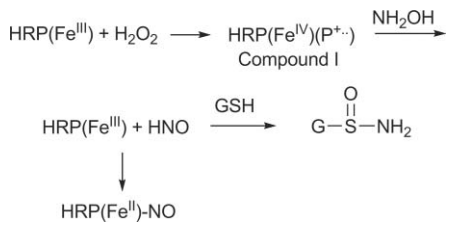
to L-*N*-hydroxyarginine to nitric oxide, which is catalyzed by nitric oxide synthase (NOS), provides a potential HNO-forming pathway as this sequence passes through the formal HNO nitrogen oxidation state ($N = -3, -1, +1, +2$ for L-arginine, L-*N*-hydroxyarginine, HNO, and NO, respectively). In the absence of the redox co-factor tetrahydrobiopterin, NOS catalyzes the formation of HNO from L-arginine giving credence to such a route for endogenous HNO formation.[92] While the distinct and controlled biological activity observed with HNO donors suggests a natural role for HNO, the reactivity of HNO and the lack of specific detection methods hinder the confirmation of *in vivo* HNO production.

A number of N-containing substrates in lower nitrogen oxidation states (similar to L-*N*-hydroxyarginine) undergo *in vitro* heme-protein mediated oxidation with subsequent HNO formation. Studies regarding HNO release from hydroxylamine, hydroxyurea, hydroxamic acids, azide, and cyanamide will be summarized. Reductive pathways of biological HNO formation from NO exist but will not be covered.[93] Oxidative metabolism defines an alternative pathway for HNO generation and may provide clues to mechanisms of endogenous HNO formation. Catalase-mediated oxidative HNO production from cyanamide, a clinical therapy for alcoholism, provides an explanation of this drug's actions.[11] Given the rapid reactions of HNO with hemes, HNO from heme protein-mediated oxidations may further react to give other products including NO or a ferrous nitrosyl complex.

3.1 HNO from hydroxylamine

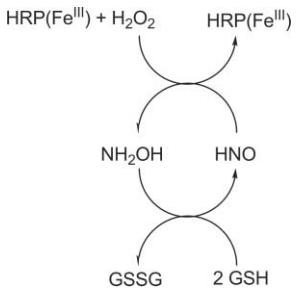
Hydroxylamine rapidly reacts with heme proteins including oxy, deoxy, and metHb to produce NO.[64,94-96] These reactions generate other nitrogenous products including nitrogen gas and ammonia but do not clearly form HNO.[97]

Treatment of hydroxylamine with HRP in the presence of hydrogen peroxide generates HNO as determined by HNO trapping with glutathione and HPLC/mass spectrometric identification of glutathione sulfinamide (Scheme 13).[63] Gas chromatographic identification of nitrous oxide in the headspace of this reaction further confirms HNO formation.[63] In this system, hydrogen peroxide activates HRP to the reactive intermediate Compound I that oxidizes hydroxylamine to HNO (Scheme 13). This HNO can reductively nitrosylate HRP(Fe^{III}) to yield HRP(Fe^{II})NO that can be detected by EPR spectroscopy and that auto-oxidizes to the ferric heme and nitrate (Path a, Scheme 13).[63] Alternatively, HNO may escape the heme pocket and react with other trapping agents (glutathione) or dimerize to form nitrous oxide (Path b, Scheme 13).[63] L-*N*-Hydroxyarginine does not form the glutathione sulfinamide or nitrous oxide under these conditions.[63] A survey of various heme proteins reveals that those with a histidine proximal ligand (HRP, myeloperoxidase, Hb and Mb) preferentially form glutathione sulfinamide from hydroxylamine giving evidence for "free" HNO in this system.[63] Proteins with proximal tyrosine/cysteine ligands (catalase/P450) generate ferrous nitrosyl complexes.[63]



Scheme 13 HRP catalyzed HNO formation from NH_2OH.

The facile peroxidase catalyzed oxidation of hydroxylamine to HNO coupled with the rapid thiol-mediated reduction of HNO to hydroxylamine forms a potential redox cycle between HNO and NH_2OH. (Schemes 1, 13, and 14).[63] Scheme 14 shows such a cycle including HRP and hydrogen peroxide as oxidants and glutathione as the reductant. By such a cycle, HNO and NH_2OH, regardless of source, could interconvert depending on local redox status possibly playing a signaling or protective role (Scheme 14).



Scheme 14 NH_2OH–HNO redox cycle.

Hydroxylamine, in the presence of catalase and a hydrogen peroxide generating system, activates soluble guanylate cyclase.[70,71] EPR spectroscopic examination of this reaction reveals the formation of the Cat(Fe^{II})NO complex,[70] which forms during the reaction of HNO and ferric catalase. Given the inability of NO to reduce ferric catalase,[42] these results strongly imply initial HNO formation followed by reductive nitrosylation. This ferrous catalase nitrosyl complex acts as an NO donor and may transfer NO to sGC leading to enzyme activation.[67] Such a system appears to represent a unique HNO-derived sGC activator and indicates HNO donor/catalase systems may be considered as NO donors.

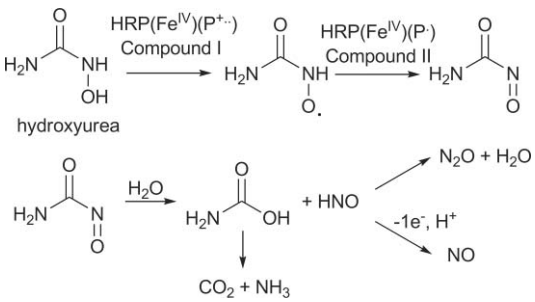
3.2 HNO from hydroxyurea and hydroxamic acids

Hydroxyurea demonstrates a diverse pharmacological profile and has a history as a human chemotherapeutic agent. Hydroxyurea remains an important treatment for a number of myeloproliferative disorders, especially chronic myelogenous leukemia.[98] Hydroxyurea is also an approved therapy for sickle cell disease, and a long-term study indicates that hydroxyurea therapy reduces mortality 40%.[99] Hydroxyurea also acts as a source of NO, which has drawn considerable interest as a sickle cell disease treatment.[100,101] Multiple lines of evidence indicate the *in vivo* conversion of hydroxyurea to NO in sickle cell disease patients.[102,103]

Hydroxyurea reacts with oxy, deoxy, and metHb *in vitro* to form iron nitrosyl hemoglobin but these reactions do not occur fast enough to account for NO production observed in patients undergoing sickle cell therapy.[104,105] To reconcile these observations, alternative mechanisms of NO formation from hydroxyurea have been considered including: 1) peroxidase or catalase mediated formation of NO from hydroxyurea and 2) NO production after hydrolysis of hydroxyurea to hydroxylamine. In the presence of urease, which catalyzes the hydrolysis of

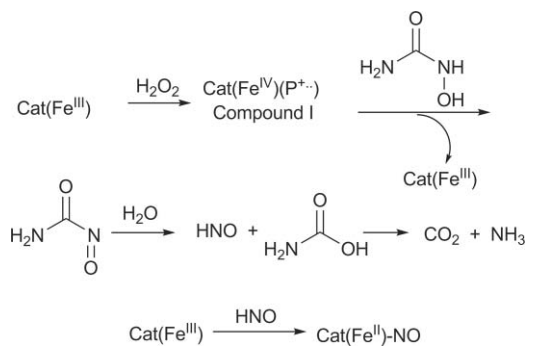
hydroxyurea to hydroxylamine, incubation of hydroxyurea with hemoglobin rapidly forms iron nitrosyl hemoglobin.[106]

In the presence of hydrogen peroxide, HRP catalyzes the rapid formation of HNO and NO from hydroxyurea.[62] Scheme 15 depicts a proposed mechanistic model that accounts for HNO and NO formation as well as the observed nitroxide radical and acyl nitroso intermediates during this reaction.[62] Hydrogen peroxide oxidation of HRP produces the reactive intermediate Compound I that acts as a one-electron oxidant of hydroxyurea to give the nitroxide radical and Compound II.[62] Further one-electron oxidation by Compound II yields the acyl nitroso compound and resting ferric HRP (Scheme 15).[62] Acyl nitroso compound hydrolysis gives carbamic acid that decomposes to carbon dioxide, ammonia, and HNO, which dimerizes and dehydrates to nitrous oxide (Scheme 15).[62] Further single electron oxidation of HNO by the ferric heme of HRP provides a route for NO production. As NO acts as a substrate for both Compounds I and II of HRP, these reactive intermediates may also act as potential single electron oxidants of HNO.[107] These results show that this HRP system rapidly converts hydroxyurea to HNO through the intermediacy of an acyl nitroso compound intermediate and identifies peroxidases as potential oxidants for the *in vivo* conversion of hydroxyurea to HNO and NO.



Scheme 15 HRP catalyzed HNO formation from hydroxyurea.

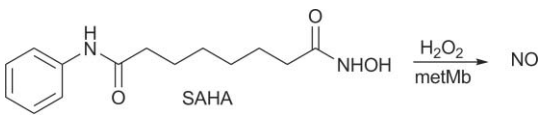
Similar to HRP, catalase in the presence of a hydrogen peroxide generating system converts hydroxyurea to HNO and NO.[67] EPR spectroscopy shows that under these conditions, hydroxyurea and catalase produce the ferrous-NO catalase complex by the appearance of the characteristic triplet signal at $g = 1.99$ in the EPR spectra.[67] Scheme 16 outlines the proposed mechanism for HNO and cat(Fe^{II})NO formation during the reaction with hydroxyurea. Hydrogen peroxide activates ferric catalase to Compound I and direct two-electron oxidation of hydroxyurea yields the acyl nitroso compound intermediate.[67] These results and the previously described HRP experiments provide evidence for enzyme catalyzed formation of an acyl nitroso compound. Unlike the reaction of hydroxyurea and HRP, gas chromatographic headspace analysis does not show the presence of nitrous oxide supporting ferric heme trapping of HNO.[67] In addition, room temperature EPR measurements fail to identify the nitroxide radical of hydroxyurea supporting a direct two-electron oxidation.[67] Hydrolysis of the acyl nitroso compound yields HNO and carbamic acid that decomposes to carbon dioxide and ammonia.[67] Reductive nitrosylation of the ferric heme of catalase by HNO gives the ferrous NO-catalase complex.



Scheme 16 Cat catalyzed HNO formation from hydroxyurea.

These results, combined with those from HRP, describe a defined pathway of HNO formation from hydroxyurea and oxidative ferric heme proteins. Two-electron oxidation of hydroxyurea produces the acyl nitroso compound that hydrolyzes to HNO and carbamic acid. Nitroxyl formed in these reactions can 1) escape the heme pocket (HRP) or 2) reductively nitrosylate the ferric heme to yield ferrous-NO complexes (Cat). The structural differences controlling this reactivity remain undefined at this time. The ferrous-NO complexes formed in these Cat reactions appear to act as NO donors capable of sGC activation. Such work identifies acyl nitroso compounds as HNO donors and HNO/ferric heme protein couples as NO sources depending on the protein.

Hydroxamic acids (RCONHOH) demonstrate diverse biological activities primarily based upon their metal chelating properties.[108] These agents find use for iron overload and cancer treatment with suberoylanilide hydroxamate (SAHA, a histone deacetylase inhibitor) being approved for cancer treatment.[109] Hydroxamic acids bear structural similarity to hydroxyurea, and hydrolysis of these carboxylic acid derivatives yields hydroxylamine.[110] While these properties make hydroxamic acids ideal candidates for heme protein-mediated HNO formation, little work in this area has appeared. A recent paper reports treatment of SAHA with hydrogen peroxide and metMb produces NO and the authors suggest the intermediacy of HNO but further mechanistic characterization is not described (Scheme 17).[111] Given the development and use of hydroxamic acids as therapeutic agents and their direct hydrolysis to hydroxylamine, a better understanding of their HNO-producing metabolism appears warranted.



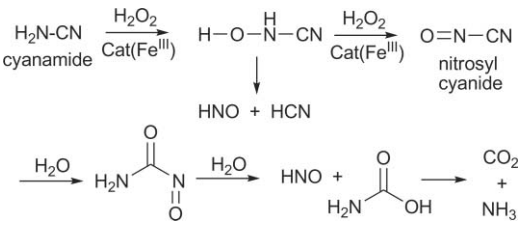
Scheme 17 NO formation from SAHA.

3.3 HNO from cyanamide

Cyanamide (H_2N-CN) has been used for many years to treat alcoholism around the world.[2] Cyanamide elicits its effects by acting as an HNO prodrug and requires bioactivation by catalase.[11] Cyanamide-derived HNO modifies the active site thiol of aldehyde dehydrogenase inhibiting normal ethanol metabolism.[10] Studies with purified enzyme show both a reversible and irreversible component to HNO inhibition of aldehyde dehydrogenase.[10] The reversible portion of inhibition has been attributed to disulfide formation and the irreversible inhibition from HNO-mediated

sulfinamide formation.[10] While metabolic activation of cyanamide also forms the cyanide ion, evidence of cyanide toxicity in cyanamide use has not been reported.[2]

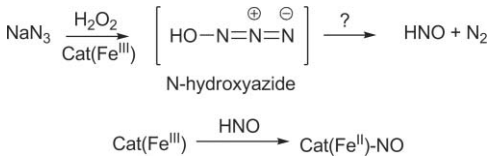
Detailed chemical and biochemical studies outline the mechanistic pathways of HNO release from cyanamide.[10,11,112] Treatment of cyanamide with catalase and a hydrogen peroxide generating system forms unstable *N*-hydroxycyanamide through a unique catalase-mediated *N*-hydroxylation (Scheme 18). *N*-Hydroxycyanamide can directly decompose to HNO and HCN (Path a) or be further oxidized by catalase to nitrosyl cyanide (ONCN, Path b).[112] Nitrile hydrolysis of nitrosyl cyanide gives the same acyl nitroso species observed in the HRP and cat-mediated oxidations of hydroxyurea and hydrolysis yields HNO and carbamic acid that decomposes to carbon dioxide and ammonia (Scheme 18).[112] Gas chromatographic/mass spectrometric analysis and NMR spectroscopy provide evidence for HNO, carbon dioxide, and cyanide formation in these studies.[11,112] The identification of nitrous oxide and the modification of thiols in aldehyde dehydrogenase clearly indicate the generation of "free" HNO in this system and no evidence exists for HNO trapping by the catalase ferric heme to give cat(Fe^{II})NO.[10,11,73] These results directly contrast those with the catalase/hydrogen peroxide mediated oxidations of hydroxylamine and hydroxyurea which form cat(Fe^{II})NO but do not generate nitrous oxide.[67] One may speculate a distinguishing role for the cyanide ion through binding to ferric catalase and blocking reductive nitrosylation allowing HNO diffusion. The chemical, biochemical, and clinical body of work regarding cyanamide shows the feasibility of HNO donors as therapeutic agents and supports the development of other HNO donor systems.



Scheme 18 Cat catalyzed HNO formation from cyanamide.

3.4 HNO from sodium azide

Sodium azide has long been recognized to possess vasorelaxant properties.[94,113] These actions occur through the activation of sGC *via* the intermediacy of NO, similar to other nitrovasodilators.[74,113,114] Sodium azide's activity requires oxidative metabolic activation to NO and the mixture of sodium azide, catalase, and a hydrogen peroxide generating system forms cat(Fe^{II})NO and competently activates sGC.[70] The inability of NO to directly reduce cat(Fe^{III}) strongly implies HNO involvement in the catalase-mediated oxidation of sodium azide.[42] As in the other systems described, cat(Fe^{II})NO appears to act as a NO donor able to activate sGC. A catalase mediated *N*-hydroxylation similar to that described for cyanamide may yield an *N*-hydroxyazide intermediate that decomposes to HNO and nitrogen (Scheme 19), which likely generates NO through the intermediacy of HNO and a cat(Fe^{II})NO. This system requires more study to distinguish



Scheme 19 Proposed HNO formation from azide.

whether azide generates both NO and HNO or simply one of the nitrogen monoxides.

Conclusions

Nitroxyl (HNO), one-electron reduced and protonated NO, demonstrates biological activities distinct from nitric oxide (NO) including the inhibition of alcohol metabolism, vasorelaxation, enhanced cardiac muscle contractility, and the inhibition of glycolysis prompting consideration of these HNO donors as therapies for alcoholism, congestive heart failure, and cancer. Chemically, HNO reacts differently than NO by reacting as an electrophile with various biological nucleophiles (particularly sulfur-containing proteins). Like NO, HNO also reacts with iron heme proteins. HNO reacts with Mb(Fe^{II}) to give an anaerobically stable Mb(Fe^{II})HNO complex and reductively nitrosylates Mb(Fe^{III}) to form Mb(Fe^{II})NO. HNO reacts with Mb(Fe^{II})O_2 in a reaction that involves the oxygen ligand to give Mb(Fe^{III}) and NO. Currently, these reactions with the common forms of Hb or Mb provide a basis to understand HNO reactions with other heme proteins. In general, other iron containing heme-proteins react with HNO similar to Hb and Mb.

The study of HNO chemistry, biology, and therapeutics requires chemical HNO donors as endogenous HNO formation that has yet to be clearly defined. We summarize the oxidative heme protein-mediated metabolism of various nitrogen substrates (hydroxylamine, hydroxyurea, hydroxamic acids, cyanamide, and sodium azide). Each of these compounds require oxidative activation by a heme protein, generally cat or a peroxidase, to form HNO. These processes generate higher oxidation state enzyme intermediates (Compounds I and II) that convert the substrate to HNO or an unstable HNO-forming intermediate. The nascent HNO may react with the ferric heme to yield a ferrous nitrosyl complex or may escape the heme pocket as HNO. Such work defines an alternative oxidative metabolic mechanism of HNO formation compared to strict chemical donors and may provide an insight into potential endogenous HNO formation.

References

1 L. J. Ignarro, ed., *Nitric Oxide: Biology and Pathobiology*, Academic Press, San Diego, 2000.
2 J. M. Fukuto, C. L. Bianco and T. A. Chavez, *Free Radical Biol. Med.*, 2009, **47**, 1318–1324.
3 J. C. Irvine, R. H. Ritchie, J. L. Favaloro, K. L. Andrews, R. E. Widdop and B. K. Kemp-Harper, *Trends Pharmacol. Sci.*, 2008, **29**, 601–608.
4 N. Paolocci, M. I. Jackson, B. E. Lopez, K. Miranda, C. G. Tocchetti, D. A. Wink, A. J. Hobbs and J. M. Fukuto, *Pharmacol. Ther.*, 2007, **113**, 442–458.
5 K. M. Miranda, *Coord. Chem. Rev.*, 2005, **249**, 433–455.
6 F. T. Bonner and M. N. Hughes, *Comments Inorg. Chem.*, 1988, **7**, 215–234.
7 K. M. Miranda, H. T. Nagasawa and J. P. Toscano, *Curr. Top. Med. Chem.*, 2005, **5**, 649–664.

8 S. B. King and H. T. Nagasawa, in *Nitric Oxide, Pt C*, 1999, **301**, 211-220.
9 D. A. Bazylinski and T. C. Hollocher, *Inorg. Chem.*, 1985, **24**, 4285–4288.
10 E. G. DeMaster, B. Redfern and H. T. Nagasawa, *Biochem. Pharmacol.*, 1998, **55**, 2007–2015.
11 H. T. Nagasawa, E. G. Demaster, B. Redfern, F. N. Shirota and J. W. Goon, *J. Med. Chem.*, 1990, **33**, 3120–3122.
12 J. M. Fukuto, K. Chiang, R. Hszieh, P. Wong and G. Chaudhuri, *J. Pharmacol. Exp. Ther.*, 1992, **263**, 546–551.
13 N. Paolocci, T. Katori, H. C. Champion, M. E. St John, K. M. Miranda, J. M. Fukuto, D. A. Wink and D. A. Kass, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 5537–5542.
14 N. Paolocci, W. F. Saavedra, K. M. Miranda, C. Martignani, T. Isoda, J. M. Hare, M. G. Espey, J. M. Fukuto, M. Feelisch, D. A. Wink and D. A. Kass, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 10463–10468.
15 C. G. Tocchetti, W. Wang, J. P. Froehlich, S. Huke, M. A. Aon, G. M. Wilson, G. Di Benedetto, B. O'Rourke, W. D. Gao, D. A. Wink, J. P. Toscano, M. Zaccolo, D. M. Bers, H. H. Valdivia, H. P. Cheng, D. A. Kass and N. Paolocci, *Circ. Res.*, 2007, **100**, 96–104.
16 T. Y. Dai, Y. Tian, C. G. Tocchetti, T. Katori, A. M. Murphy, D. A. Kass, N. Paolocci and W. D. Gao, *J. Physiol.*, 2007, **580**, 951–960.
17 P. Pagliaro, *Life Sci.*, 2003, **73**, 2137–2149.
18 X. J. He, I. Azarov, A. Jeffers, T. Presley, J. Richardson, S. B. King, M. T. Gladwin and D. B. Kim-Shapiro, *Free Radical Biol. Med.*, 2008, **44**, 1420–1432.
19 B. E. Lopez, C. E. Rodriguez, M. Pribadi, N. M. Cook, M. Shinyashiki and J. M. Fukuto, *Arch. Biochem. Biophys.*, 2005, **442**, 140–148.
20 B. E. Lopez, D. A. Wink and J. M. Fukuto, *Arch. Biochem. Biophys.*, 2007, **465**, 430–436.
21 A. J. Norris, M. R. Sartippour, M. Lu, T. Park, J. Y. Rao, M. I. Jackson, J. M. Fukuto and M. N. Brooks, *Int. J. Cancer*, 2008, **122**, 1905–1910.
22 L. Chazotte-Aubert, S. Oikawa, I. Gilibert, F. Bianchini, S. Kawanishi and H. Ohshima, *J. Biol. Chem.*, 1999, **274**, 20909–20915.
23 J. Ivanova, G. Salama, R. M. Clancy, N. F. Schor, K. D. Nylander and D. A. Stoyanovsky, *J. Biol. Chem.*, 2003, **278**, 42761–42768.
24 C. A. Colton, M. Gbadegesin, D. A. Wink, K. M. Miranda, M. G. Espey and S. Vicini, *J. Neurochem.*, 2001, **78**, 1126–1134.
25 C. U. Choe, J. Lewerenz, G. Fischer, T. F. Uliasz, M. G. Espey, F. C. Hummel, S. B. King, E. Schwedhelm, R. H. Boger, C. Gerloff, S. J. Hewett, T. Magnus and S. Donzelli, *J. Neurochem.*, 2009, **110**, 1766–1773.
26 V. Shafirovich and S. V. Lymar, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 7340–7345.
27 M. D. Bartberger, J. M. Fukuto and K. N. Houk, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 2194–2198.
28 M. D. Bartberger, W. Liu, E. Ford, K. M. Miranda, C. Switzer, J. M. Fukuto, P. J. Farmer, D. A. Wink and K. N. Houk, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 10958–10963.
29 V. Shafirovich and S. V. Lymar, *J. Am. Chem. Soc.*, 2003, **125**, 6547–6552.
30 D. M. Lemal and T. W. Rave, *J. Am. Chem. Soc.*, 1965, **87**, 393–394.
31 M. P. Doyle, S. N. Mahapatro, R. D. Broene and J. K. Guy, *J. Am. Chem. Soc.*, 1988, **110**, 593–599.
32 J. A. Reisz, E. B. Klorig, M. W. Wright and S. B. King, *Org. Lett.*, 2009, **11**, 2719–2721.
33 R. N. Dixon, *J. Chem. Phys.*, 1996, **104**, 6905–6906.
34 B. E. Lopez, M. Shinyashiki, T. H. Han and J. M. Fukuto, *Free Radical Biol. Med.*, 2007, **42**, 482–491.
35 K. M. Miranda, N. Paolocci, T. Katori, D. D. Thomas, E. Ford, M. D. Bartberger, M. G. Espey, D. A. Kass, M. Feelisch, J. M. Fukuto and D. A. Wink, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 9196–9201.
36 K. M. Miranda, M. G. Espey, K. Yamada, M. Krishna, N. Ludwick, S. Kim, D. Jourd'heuil, M. B. Grisham, M. Feelisch, J. M. Fukuto and D. A. Wink, *J. Biol. Chem.*, 2001, **276**, 1720–1727.
37 M. P. Doyle, R. A. Pickering and B. R. Cook, *J. Inorg. Biochem.*, 1983, **19**, 329–338.
38 S. Herold, M. Exner and T. Nauser, *Biochemistry*, 2001, **40**, 3385–3395.
39 R. Cassoly and Q. H. Gibson, *J. Mol. Biol.*, 1975, **91**, 301–313.
40 V. S. Sharma, R. A. Isaacson, M. E. John, M. R. Waterman and M. Chevion, *Biochemistry*, 1983, **22**, 3897–3902.
41 V. S. Sharma, T. G. Traylor, R. Gardiner and H. Mizukami, *Biochemistry*, 1987, **26**, 3837–3843.
42 M. Hoshino, M. Maeda, R. Konishi, H. Seki and P. C. Ford, *J. Am. Chem. Soc.*, 1996, **118**, 5702–5707.
43 S. N. Mahapatro and M. P. Doyle, *J. Am. Chem. Soc.*, 1984, **106**, 3678–3679.
44 P. J. Farmer and F. Sulc, *J. Inorg. Biochem.*, 2005, **99**, 166–184.
45 D. A. Bazylinski and T. C. Hollocher, *J. Am. Chem. Soc.*, 1985, **107**, 7982.
46 R. Lin and P. J. Farmer, *J. Am. Chem. Soc.*, 2000, **122**, 2393–2394.
47 F. Sulc, E. Fleischer, P. J. Farmer, D. Ma and G. N. La Mar, *JBIC, J. Biol. Inorg. Chem.*, 2003, **8**, 348–352.
48 F. Sulc, C. E. Immoos, D. Pervitsky and P. J. Farmer, *J. Am. Chem. Soc.*, 2004, **126**, 1096–1101.
49 M. R. Kumar, D. Pervitsky, L. Chen, T. Poulos, S. Kundu, M. S. Hargrove, E. J. Rivera, A. Diaz, J. L. Colon and P. J. Farmer, *Biochemistry*, 2009, **48**, 5018–5025.
50 J. Beetlestone and P. George, *Biochemistry*, 1964, **3**, 707–714.
51 C. D. Coryell, F. Stitt and L. Pauling, *J. Am. Chem. Soc.*, 1937, **59**, 633–642.
52 K. M. Miranda, R. W. Nims, D. D. Thomas, M. G. Espey, D. Citrin, M. D. Bartberger, N. Paolocci, J. M. Fukuto, M. Feelisch and D. A. Wink, *J. Inorg. Biochem.*, 2003, **93**, 52–60.
53 D. A. Bazylinski, J. Goretski and T. C. Hollocher, *J. Am. Chem. Soc.*, 1985, **107**, 7986.
54 M. A. Sharpe and C. E. Cooper, *Biochem. J.*, 1998, **332**(Pt 1), 9–19.
55 J. S. Stamler, D. J. Singel and J. Loscalzo, *Science*, 1992, **258**, 1898–1902.
56 A. J. Gow and J. S. Stamler, *Nature*, 1998, **391**, 169–173.
57 F. Boccini, A. S. Domazou and S. Herold, *J. Phys. Chem. A*, 2006, **110**, 3927–3932.
58 F. Azizi, J. E. Kielbasa, A. M. Adeyiga, R. D. Maree, M. Frazier, M. Yakubu, H. Shields, S. B. King and D. B. Kim-Shapiro, *Free Radical Biol. Med.*, 2005, **39**, 145–151.
59 B. Benko and N. T. Yu, *Proc. Natl. Acad. Sci. U. S. A.*, 1983, **80**, 7042–7046.
60 C. E. Cooper, *Biochim. Biophys. Acta, Bioenerg.*, 1999, **1411**, 290–309.
61 P. R. Rich and M. Iwaki, *Biochemistry (Moscow)*, 2007, **72**, 1047–1055.
62 J. M. Huang, E. M. Sommers, D. B. Kim-Shapiro and S. B. King, *J. Am. Chem. Soc.*, 2002, **124**, 3473–3480.
63 S. Donzelli, M. G. Espey, W. Flores-Santana, C. H. Switzer, G. C. Yeh, J. M. Huang, D. J. Stuehr, S. B. King, K. M. Miranda and D. A. Wink, *Free Radical Biol. Med.*, 2008, **45**, 578–584.
64 S. B. King, *Curr. Top. Med. Chem.*, 2005, **5**, 665–673.
65 P. Nicholls, *Experientia*, 1963, **19**, 80–82.
66 B. Jerónimo, J. M. Maria, S. Thomas, S. Jack, W. Keith, C. L. Peter and F. Ignacio, *Proteins: Struct., Funct., Genet.*, 1999, **34**, 155–166.
67 J. M. Huang, D. B. Kim-Shapiro and S. B. King, *J. Med. Chem.*, 2004, **47**, 3495–3501.
68 V. Massey and P. Hemmerich, *Biochemistry*, 1978, **17**, 9–16.
69 N. Shimizu, K. Kobayashi and K. Hayashi, *J. Biochem.*, 1988, **104**, 136–140.
70 P. A. Craven, F. R. Derubertis and D. W. Pratt, *J. Biol. Chem.*, 1979, **254**, 8213–8222.
71 L. J. Ignarro, J. B. Adams, P. M. Horwitz and K. S. Wood, *J. Biol. Chem.*, 1986, **261**, 4997–5002.
72 J. M. Fukuto, P. Gulati and H. T. Nagasawa, *Biochem. Pharmacol.*, 1994, **47**, 922–924.
73 M. Shahidullah, A. Duncan, P. D. Strachan, K. M. Rafique, S. L. Ball, M. J. W. McPate, S. Nelli and W. Martin, *Eur. J. Pharmacol.*, 2002, **435**, 93–101.
74 S. Katsuki, W. Arnold, C. Mittal and F. Murad, *J. Cyclic Nucleotide Res.*, 1977, **3**, 23–35.
75 Y. Naruta, T. Sasaki, F. Tani, Y. Tachi, N. Kawato and N. Nakamura, *J. Inorg. Biochem.*, 2001, **83**, 239–246.
76 S. Shiva, J. H. Crawford, A. Ramachandran, E. K. Ceaser, T. Hillson, P. S. Brookes, R. P. Patel and V. M. Darley-Usmar, *Biochem. J.*, 2004, **379**, 359–366.
77 J. M. Fukuto, C. H. Switzer, K. M. Miranda and D. A. Wink, *Annu. Rev. Pharmacol. Toxicol.*, 2005, **45**, 335–355.
78 S. I. Liochev and I. Fridovich, *Arch. Biochem. Biophys.*, 2002, **402**, 166–171.
79 F. T. Bonner, M. N. Hughes, R. K. Poole and R. I. Scott, *Biochim. Biophys. Acta, Bioenerg.*, 1991, **1056**, 133–138.
80 A. J. Hobbs, *Trends Pharmacol. Sci.*, 1997, **18**, 484–491.

81 C. Griffiths, V. Wykes, T. C. Bellamy and J. Garthwaite, *Mol. Pharmacol.*, 2003, **64**, 1349–1356.
82 T. L. Poulos, *Curr. Opin. Struct. Biol.*, 2006, **16**, 736–743.
83 J. L. Favaloro and B. K. Kemp-Harper, *Cardiovasc. Res.*, 2007, **73**, 587–596.
84 J. L. Favaloro and B. K. Kemp-Harper, *Am. J. Physiol. Heart Circ. Physiol.*, 2009, **296**, H1274–1280.
85 T. W. Miller, M. M. Cherney, A. J. Lee, N. E. Francoleon, P. J. Farmer, S. B. King, A. J. Hobbs, K. M. Miranda, J. N. Burstyn and J. M. Fukuto, *J. Biol. Chem.*, 2009, **284**, 21788–21796.
86 A. Zeller, M. V. Wenzl, M. Beretta, H. Stessel, M. Russwurm, D. Koesling, K. Schmidt and B. Mayer, *Mol. Pharmacol.*, 2009, **76**, 1115–1122.
87 J. C. Irvine, J. L. Favaloro, R. E. Widdop and B. K. Kemp-Harper, *Hypertension*, 2007, **49**, 885–892.
88 D. F. Lewis and J. M. Pratt, *Drug Metab. Rev.*, 1998, **30**, 739–786.
89 D. F. Lewis, *Pharmacogenomics*, 2003, **4**, 387–395.
90 D. A. Wink, Y. Osawa, J. F. Darbyshire, C. R. Jones, S. C. Eshenaur and R. W. Nims, *Arch. Biochem. Biophys.*, 1993, **300**, 115–123.
91 D. A. Wink and J. B. Mitchell, *Free Radical Biol. Med.*, 1998, **25**, 434–456.
92 C. C. Wei, Z. Q. Wang, C. Hemann, R. Hille and D. J. Stuehr, *J. Biol. Chem.*, 2003, **278**, 46668–46673.
93 I. M. Wasser, S. de Vries, P. Moenne-Loccoz, I. Schroder and K. D. Karlin, *Chem. Rev.*, 2002, **102**, 1201–1234.
94 P. G. Wang, M. Xian, X. P. Tang, X. J. Wu, Z. Wen, T. W. Cai and A. J. Janczuk, *Chem. Rev.*, 2002, **102**, 1091–1134.
95 V. L. Lockamy, H. Shields, D. B. Kim-Shapiro and S. B. King, *Biochim. Biophys. Acta, Gen. Subj.*, 2004, **1674**, 260–267.
96 K. Stolze and H. Nohl, *Biochem. Pharmacol.*, 1989, **38**, 3055–3059.
97 D. A. Bazylinski, R. A. Arkowitz and T. C. Hollocher, *Arch. Biochem. Biophys.*, 1987, **259**, 520–526.
98 R. C. Donehower, *Hydroxyurea*, Lippincott-Raven, Philadelphia, 1996.
99 M. H. Steinberg, F. Barton, S. Castro, C. H. Pegelow, S. K. Ballas, A. Kutlar, E. Orringer, R. Bellevue, N. Olivieri, M. Varma, G. Ramirez, B. Adler, W. Smith, T. Carlos, K. Ataga, L. DeCastro, C. Bigelow, Y. Saunthararajah, M. Teller, E. Vichinsky, S. Claster, S. Shurin, K. Bridges, M. Waclawiw, D. Bonds and M. Terrin, *JAMA, J. Am. Med. Assoc.*, 2003, **289**, 1645–1651.
100 C. Halsey and I. A. G. Roberts, *Br. J. Haematol.*, 2003, **120**, 177–186.
101 C. D. Reiter and M. T. Gladwin, *Curr. Opin. Hematol.*, 2003, **10**, 99–107.
102 M. T. Gladwin, J. H. Shelhamer, F. P. Ognibene, M. E. Pease-Fye, J. S. Nichols, B. Link, D. B. Patel, M. A. Jankowski, L. K. Pannell, A. N. Schechter and G. P. Rodgers, *Br. J. Haematol.*, 2002, **116**, 436–444.
103 M. Nahavandi, F. Tavakkoli, M. Q. Wyche, E. Perlin, W. P. Winter and O. Castro, *Br. J. Haematol.*, 2002, **119**, 855–857.
104 S. B. King, *Curr. Med. Chem.*, 2003, **10**, 437–452.
105 J. Huang, S. B. Hadimani, J. W. Rupon, S. K. Ballas, D. B. Kim-Shapiro and S. B. King, *Biochemistry*, 2002, **41**, 2466–2474.
106 V. L. Lockamy, J. M. Huang, H. Shields, S. K. Ballas, S. B. King and D. B. Kim-Shapiro, *Biochim. Biophys. Acta, Gen. Subj.*, 2003, **1622**, 109–116.
107 R. E. Glover, V. Koshkin, H. B. Dunford and R. P. Mason, *Nitric Oxide*, 1999, **3**, 439–444.
108 R. Codd, *Coord. Chem. Rev.*, 2008, **252**, 1387–1408.
109 S. Grant, C. Easley and P. Kirkpatrick, *Nat. Rev. Drug Discovery*, 2007, **6**, 21–22.
110 M. Flipo, J. Charton, A. Hocine, S. Dassonneville, B. Deprez and R. Deprez-Poulain, *J. Med. Chem.*, 2009, **52**, 6790–6802.
111 Y. Samuni, W. Flores-Santana, M. C. Krishna, J. B. Mitchell and D. A. Wink, *Free Radical Biol. Med.*, 2009, **47**, 419–423.
112 F. N. Shirota, D. J. W. Goon, E. G. DeMaster and H. T. Nagasawa, *Biochem. Pharmacol.*, 1996, **52**, 141–147.
113 H. Kimura, C. K. Mittal and F. Murad, *J. Biol. Chem.*, 1975, **250**, 8016–8022.
114 C. K. Mittal, H. Kimura and F. Murad, *J. Biol. Chem.*, 1977, **252**, 4384–4390.

