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Importance of NO and its related compounds in enteric nervous system regulation of gut homeostasis and disease susceptibility

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Abstract

Nitric oxide (NO) produced by the enteric nervous system represents an important regulatory mechanism in gut homeostasis. Aberrant NO signaling contributes significantly towards enteric disease by altering gut motility, vascular tone, blood supply, mucosal barrier function, secretions and immunity. Consequently, there is much interest in therapeutically targeting NO production and its bioactive intermediates. This article highlights recent advances in NO signaling and therapeutics as it relates to the gastrointestinal tract and its associated NO producing microbiota. Because of its limited scope, a particular emphasis is placed on S-nitrosylation as the emerging physiologic mechanism for NO signal transduction, and how such signals are modulated by other gaseous transmitters - notably hydrogen disulfide and carbon monoxide – that are produced by the enteric nervous system and share common molecular targets. Recent findings also indicate that druggable regulators of S-nitrosylation, for example S-nitrosoglutathione (GSNO) reductase, provide for a superior pharmacology and finer therapeutic control over classical NO donors, and may be better suited for oral delivery to the gastrointestinal tract.

Keywords

S-nitrosylation; nitric oxide; gastrointestinal disease; enteric nervous system; microbiota; hydrogen sulfide; carbon monoxide; NO therapeutics

Introduction

Unprecedented interest in NO related biology was spurred three decades ago with the discovery of NO as a novel gaseous signaling molecule in intracellular and intercellular communications^{1,2}. Pathogenic NO production is now universally recognized to play a major role in disease etiology, and justifiably there is intense interest in targeting NO for therapeutic intervention. Enteric disease is no exception to the effects of aberrant NO production³. For example, in patients with esophageal achalasia loss of inhibitory nitric

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neurons causes difficulty in swallowing, regurgitation and chest pain⁴. In premature infants with necrotizing enterocolitis, loss of nitrergic neurons is associated with clinical onset⁵, and the pathologic sequelae are recapitulated in animal models after targeted ablation of NO producing cells in the enteric nervous system^{6–8}. Similarly, targeted deletion of specific enzymes of the NO synthesis machinery⁹ or of downstream molecular effectors of NO signal transduction such as soluble guanylyl cyclase¹⁰, results in a wide range of gastrointestinal (GI) pathology, including fulminant inflammation and cancer, motility defects and susceptibility to infection. These findings clearly demonstrate a physiologic role for NO in regulating gut homeostasis, and although clinical symptoms may be alleviated by exogenous NO donors, therapeutic targets remain poorly defined. This article provides a current perspective of how NO signals from multiple diverse sources transduce bioactivity in the GI tract, and how modulators of such signaling pathways represent new druggable targets in NO therapeutics.

Enteric Sources of Regulatory NO

P450-like mammalian NO synthases (NOS) are generally regarded as the predominant enzymatic source of NO from L-arginine conversion, and normally require oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) co-substrates¹¹. Several genetically distinct NOS isoenzymes exist, although in the enteric nervous system neuronal (NOS1) and inducible (NOS2) isoforms constitute the major cellular sources of NO release from enteric neurons and glia respectively, playing important regulatory roles in enteric synaptic transmission, muscular tone, mucosal barrier function and fluid secretion¹². Both intrinsic and environmental cues fine tune NO synthesis, and include calcium-regulated activation of NOS2 in enteric glia by purine and nicotinamine agonists¹³, and formation of enzymatically active NOS1 homodimers in enteric neurons by cofactors heme, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin¹⁴. Interestingly, enteric glia are emerging as a major regulatory source of NO that exert wide ranging protective functions in the GI tract, in part via production of reactive S-nitrosothiol intermediates¹⁵ (see *Enteric NO Signaling Mechanisms*). Such protective effects do not require close proximity to targets cells, for example NOS2 activity in enteric glia in the myenteric plexus appears to have evolved as a novel anti-secretory signal to the colonic mucosa, thereby restricting excessive fluid secretion during GI disease states¹⁶.

The GI tract is unique in that large amounts of bioactive NO is also generated from dietary sources via NOS-independent chemical reaction of dietary nitrite with hydrochloric acid in the stomach^{17,18}, and by microbial reduction following aerobic conversion in the oral cavity¹⁹, or by anaerobic reaction with nitrate in the colon by *Escherichia coli* spp²⁰. Entero-salivary nitrate conversion pathways also provide a rich source of bioactive NO, and nitrate-reducing bacteria such as *Veillonella* and *Actinomyces* spp have been identified as active NO producers in the oral mucosa¹⁹. Microbial dysbiosis associated with enteric disease may therefore represent a previously unappreciated player in NO homeostasis in the GI tract, and supplementing and/or targeting NO generating bacterial species represents a potentially novel therapeutic approach²¹.

Enteric NO Signaling Mechanisms

NO signals via classic cyclic GMP (cGMP)-dependent or more recently discovered cGMP-independent pathways. In the classical signaling pathway, soluble guanylyl cyclase activity is elicited by NO following binding to its heme cofactor center, resulting in elevated cGMP concentrations. This intracellular second messenger signal then activates several cGMP-dependent protein kinases, phosphodiesterases and ion channels that transduce the NO bioactivity. The reader is referred to a number of excellent reviews on cGMP-mediated NO signaling^{22–25}.

It is now increasingly appreciated that cGMP-independent signaling mechanisms have also emerged as major physiologic conduits of NO bioactivity in CNS, cardiovascular, immune, respiratory and enteric systems²⁶. NO exists in a number of intermediate states that can form chemical adducts with biological macromolecules, especially under conditions of oxidative stress when highly reactive peroxynitrite (ONOO[−]), nitrosium (NO⁺), nitrogen dioxide (NO₂) and dinitrogen trioxide (N₂O₃) are formed²⁷. Redox-sensitive thiol groups on cysteine residues (Cys) are prone to NO adduct formation. Because such cysteine residues are often strategically positioned to regulate protein function and/or stability, the addition of an NO adduct may drastically alter its biomolecular function, for example by inhibiting catalytic activity in cysteine proteases²⁸. Unlike peroxynitrite which is preferentially formed during oxidative stress and is associated with cellular damage, S-nitrosylation is a reversible, labile posttranslational modification of a protein or peptide Cys by NO, forming an S-nitrosothiol (SNO) as shown in Figure 1.

Because S-nitrosylation targets proteins in virtually all known cellular pathways under steady state conditions - ranging from diverse functions such as ion channel modification, inhibitory protein-DNA interactions, G-protein coupled receptor activation - this posttranslational modification is gaining widespread recognition as the NO signaling equivalent to O-phosphorylation and ubiquitylation (regulated by protein kinases and ubiquitin E3 ligases, respectively)²⁹. Contrary to commonly stated views in the literature, S-nitrosylation does not appear to randomly target redox-sensitive Cys. Rather, specific and often redox-insensitive Cys are modified and this appears to be regulated in part through electrostatic, steric factors, and allosteric modulators of protein function. For example, there are several redox-sensitive Cys in ryanodine response calcium channel of skeletal muscle, yet only one cysteine (Cys3635) forms part of an S-nitrosylation motif that selectively regulates calmodulin-dependent NO-mediated modulation of channel activity^{30,31}. Intense research interest is currently focused on understanding what Cys specificity determinants target a residue for S-nitrosylation. Computational studies of SNO-consensus motifs have identified patterns in both primary amino acid sequence (acid-base motif) and in tertiary structure (exposed acid motif)^{32–36}. Charged amino acid residues forming part of these proposed consensus motifs are thought to promote Cys reactivity to NO, or to engage in protein-protein interactions^{36,37}. In support of the view that S-nitrosylation is governed by structural motifs that promote protein-protein interactions is the recent discovery of specific nitrosylases and denitrosylases, which are now being targeted for therapeutic intervention^{37,38}. The nascent nitrosylase concept is emerging as an attractive mechanism for conferring S-nitrosylation specificity, and also offers an explanation of how

S-nitrosylated proteins can appear within cellular compartments that normally lack NOS activity. For example, translocation of glyceraldehyde 3-phosphate dehydrogenase into the nucleus after its S-nitrosylation in the cytosol has been proposed to trigger a nuclear death signal in neurons by acting as a specific nitrosylase for sirtuin 1, histone deacetylase-2, and DNA-activated protein kinase³⁹.

Further work is required to establish whether nitrosylases represent a universal S-nitrosylation mechanism. In our hands, systematic examination of all experimentally confirmed SNO-sites in the published structural PDB database has failed to demonstrate a consensus motif in tertiary structure that involves charged amino acids that could enhance Cys reactivity, decrease pK_a or promote protein-protein interactions (*unpublished findings*). Furthermore, analysis of predicted protein-protein interfaces does not support preferential interactions with SNO sites. This lack of consistency in motif bias is similar to that reported for sulfenylation (see *Enteric Modulators of NO Signaling*), and suggests that several types of S-nitrosylation mechanism may operate to transduce NO signals *in vivo*.

Because relatively few GI-based studies have investigated whether NO signals are cGMP-dependent or not, it seems likely that S-nitrosylation also plays a major regulatory role in enteric nervous system function. In support of this notion, recent studies have demonstrated that NO-mediated intestinal smooth muscle relaxation is Cys thiol-dependent⁴⁰. Further, cGMP-independent signaling is implicated as an NO regulatory mechanism of inhibitory synaptic potentials (IPSPs), slow excitatory synaptic potentials (EPSPs) and action potential firing in submucosal neurons⁴¹. We and others have demonstrated that S-nitrosylation signals are not only present in the GI tract, but actively function to maintain gut function and barrier integrity¹⁵, and to protect the host from trauma⁴² and infection^{28,43}. As a compensatory response, several enteric pathogens have evolved NO detoxification systems to counter specific host (and possibly microbiome) S-nitrosylation defenses⁴⁴.

Enteric Modulators of NO Signaling

With the discovery that NO exploits Cys and cGMP as distinct signaling messengers, modulators of thiolate and guanylyl cyclase activity provides a fundamental basis for cross-regulation⁴⁵. The enteric nervous system generates other gaseous transmitters that share signaling pathways and common biological effects with NO³. For example, hydrogen sulfide and carbon monoxide both regulate smooth muscle tone, neurotransmission and immune function, and target Cys thiol and the heme center of guanylyl cyclase, respectively (Fig. 2). Hydrogen sulfide represents an interesting example of a potential modulator of S-nitrosylation. This gas is generated by cystathionine β synthase and cystathionine α lyase in the GI tract (and also by the gut microbiota), and targets Cys thiol via sulfenylation - formation of mixed disulfides. In fact, hydrogen sulfide is reported to potentiate NO signaling, and in some instances is a requirement for NO bioactivity (possibly by releasing NO from S-nitrosothiols)^{45,46}. However, a number of physiologic attributes differ between these transmitters, and in part may be explained by differential off targets effects, the formation of stable disulfide bonds with Cys, or reflect dose-dependency. NO biology is littered with examples of dose-dependency effects.

Carbon monoxide administered therapeutically as a gas also shares many effects with NO donors, such as potentiating gut motility, protection from sepsis, and reducing intestinal inflammation³. Even though the potency of carbon monoxide for guanylyl cyclase activation is significantly lower than that for NO⁴⁷, it can achieve tissue concentrations that are sufficiently high to activate cGMP signaling and this may potentiate NO signaling, especially following induction of carbon monoxide synthesis by inducible heme oxygenase during oxidative stress. In support of this view, in the rat brain inducible heme oxygenase-derived carbon monoxide acts as a tonic regulator of NO dependent vasodilation³. Evidence that carbon monoxide can recapitulate NO bioactivity in the GI tract is also evident from the observation that the complete loss of non-adrenergic non-cholinergic neurotransmission is only achieved in mice lacking both *Nos1* and *Hmox2*, where activity can be restored by exogenous donors^{48,49}. Thus, there is potential scope to modulate and/or potentiate the therapeutic effects of NO using a combinatorial approach that also targets other gaseous transmitters.

NO therapeutics: deliverables into the GI tract

Utility of NO therapeutics in clinical practice is supported in the broader context by recognizing that hypo- and hyper-nitrosylation of specific proteins represent disease-modifying events. For example, hypo-nitrosylation of the cystic fibrosis transmembrane conductance regulator (CFTR) is associated with poor respiratory function in patients with cystic fibrosis⁵⁰. In neurodegenerative disorders of the brain, altered denitrosylating activity of mutant superoxide dismutase 1 is implicated in patients with amyotrophic lateral sclerosis^{51,52}. Loss of neurons is associated with excessive S-nitrosylation of Cys644 on dynamin-related protein 1 in Alzheimer's disease⁵³. Moreover, Parkin⁵⁴, glyceraldehyde 3-phosphate dehydrogenase, peroxiredoxin-2 and protein disulfide isomerase, appear to be other aberrantly S-nitrosylated regulatory proteins in Alzheimer's and Parkinson's disease^{39,54-56}.

A major new goal of NO therapeutics is to target and selectively control the nitrosylation state of proteins that are identified with pathophysiology. This approach is supported by the identification of specific molecular targets that are aberrantly S-nitrosylated and are clinically responsive to NO-therapeutics⁵⁷. For example, banked blood enriched in S-nitrosylated hemoglobin drastically improves oxygen delivery and is set to revolutionize transfusion medicine⁵⁸. S-nitrosothiol to promote CFTR function have demonstrated good efficacy in models of cystic fibrosis⁵⁰. Preclinical studies have also demonstrated promise for targeted S-nitrosothiol therapy in CNS and cardiovascular disease models^{59,60}. This is also evident in the GI tract where S-nitrosothiols ameliorate intestinal barrier dysfunction and acute inflammation, alter fluid and peptide secretion, correct motility defects, and modulate immune function and susceptibility to infection. In the latter case, we have demonstrated that therapeutic S-nitrosothiol attenuates certain gut bacterial infections by providing disease-attenuating signals¹⁵. In this instance, we reported the first example of how allosteric therapeutics may be applied to confer clinical efficacy using allosteric enhancers to promote targeted S-nitrosylation of *Clostridium difficile* toxins²⁸.

Finally, because S-nitrosothiols exert distal and longer lasting therapeutic effects than NO gas (*in vivo* steady state concentrations 100 pM-5 nM and 0.1–2 s half-life, with an autocrine biological range of <200 μ m), these compounds represent an interesting approach to specifically target aberrant NO homeostasis. However, there are potentially a number of short comings relating to the delivery of S-nitrosothiols, including lead compound stability, transfer dynamics of the reactive NO group, and potential off target effects. This is particularly problematic in the GI tract where the intestinal mucosa and its associated microbiota have evolved to rapidly digest small peptides and to detoxify NO in its various chemical forms. New therapeutic strategies are showing promise in overcoming such limitations by directly targeting the host NO regulatory machinery to elevate endogenous tissue S-nitrosothiol concentrations. Notably, GSNO reductase is a highly conserved catabolic enzyme that converts GSNO to 5-hydrooxylamine-glutathione, thereby maintaining intracellular homeostasis of the protein-SNO pool⁵⁷. N6022 is a potent first-in-class pyrrole-based inhibitor of GSNO reductase showing promise in clinical trials for asthma⁶¹. Mechanistically, N6022 functions by enhancing bioavailable GSNO and the intracellular SNO-protein pool⁶². Preclinical studies have also demonstrated N6022 efficacy in the GI tract when applied to treat experimental inflammatory bowel disease. Although these recent findings represent significant advances in the field, caution still needs to be exercised when evaluating the therapeutic benefits of modulating the protein-SNO pool since altering NO homeostasis is associated with potentially detrimental side effects, including susceptibility to infection, toxicity and cancer.

Conclusion

An emerging concept in NO biology is that S-nitrosothiols represent important regulatory signals that can also mediate therapeutic benefits. Preliminary studies in the enteric nervous system support this view, and implicate S-nitrosylation as an important NO regulatory signal in the GI tract. NO derived signals from enteric neurons and glia may therefore confer wide ranging protective functions and represent important target cells in GI inflammatory and permeability disorders. These regulatory signals also appear to operate in a coordinated manner with other gaseous transmitters - hydrogen sulfide and carbon monoxide – which are being independently pursued as GI therapeutics. Careful examination of cross-regulatory signals needs to be conducted to assess whether a combinatorial therapeutic approach may potentiate the clinical efficacy of NO in the GI tract. New drugs have been designed to directly manipulate endogenous protein-SNO homeostasis as a novel approach to deliver NO bioactivity without the pharmacological limitations of classical NO donors and S-nitrosothiols. Manipulation of the gut microbiota offers another relatively untapped strategy for NO therapeutics that will likely generate an extensive repertoire of specific druggable targets in the future.

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Highlights

- S-nitrosothiols mediate NO bioactivity and confer therapeutic benefits
- Cross-regulatory signals with other transmitters may potentiate NO bioactivity
- New druggable regulators of S-nitrosothiol homeostasis show therapeutic promise
- The gut microbiota represents an unexplored target for NO therapeutics

Figure 1. Protein S-nitrosylation. Analysis of crystal structures of native apoprotein versus S-nitrosylation variants demonstrate no drastic structural changes as is illustrated for four human proteins thioredoxin (oxidized form), S100A1, PTPB1, and PDH2. Upper row shows native proteins (green) and their S-nitrosylated forms (ivory) in ribbon representation. S-nitrosylation cysteine targets (C62, C85, C125, and C302) are shown in yellow (native) and cyan spheres. Bottom row shows the same proteins in their S-nitrosylated form in surface representation.

Figure 2.
Schematic outline of S-nitrosylation signals and cross-regulation with other gaseous transmitters in the GI tract.