Lessons From Genetically Engineered Animal Models IV. Nitric oxide synthase gene knockout mice*

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Mashimo, Hiroshi, and Raj K. Goyal. Lessons From Genetically Engineered Animal Models. IV. Nitric oxide synthase gene knockout mice. *Am. J. Physiol.* 277 (*Gastrointest. Liver Physiol.* 40): G745–G750, 1999.—Nitric oxide is a ubiquitous molecule implicated in a variety of biological processes. The specific action of nitric oxide depends on its enzymatic sources, namely neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), each having distinct tissue localization. Conventional pharmacological antagonists could not distinguish these enzymes or provide models of chronic nitric oxide depletion in whole animals. Several lines of knockout mice have been generated to distinguish the roles of nitric oxide from each enzyme: nitric oxide from nNOS is a major inhibitory neurotransmitter, nitric oxide from eNOS regulates blood flow under physiological conditions, and nitric oxide from iNOS causes hypotension during severe inflammatory conditions. Moreover, the nitric oxides from each isoform have different roles in tissue injury and inflammation. Studies of NOS-deficient animals have also identified redundant and compensatory pathways and revealed the consequences of life-long deficiency of these enzymes. The nNOS-deficient mice develop gastric dilation and stasis, the eNOS-deficient mice develop hypotension and lack vasodilatory responses to injury, and iNOS-deficient mice are more susceptible to inflammatory damage but more resistant to septic shock.

L-arginine; vasodilation; gut motility

SINCE THE DISCOVERY IN 1980 of nitric oxide (NO) as a potent vascular smooth muscle relaxant and regulator of blood pressure, NO has been found in many cell types and implicated in a variety of biological roles. Indeed, NO is involved in the health and disease of all organs and systems. Its diverse and bewildering biological functions in the gastrointestinal tract have been reviewed extensively (23a).

NO is a reactive and readily diffusible gas produced from L-arginine by one of three nitric oxide synthase (NOS) enzymes, namely, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Various roles of NO in the gastrointestinal tract, as in other organs, have been implicated through the use of chemical inhibitors; blockade of NOS by these compounds and its reversal by L-arginine serve as evidence that NO is involved in a specific biological function.

The role of NO in the gastrointestinal tract depends largely on its specific enzymatic source and tissue localization (Table 1). nNOS is expressed primarily in neurons, eNOS in vascular endothelium, and iNOS in macrophages. Of these, nNOS and eNOS are constitutively expressed and respond to calcium-calmodulin signaling. iNOS is induced by inflammatory mediators and is coupled to an activated calmodulin that does not require calcium for activation. These three enzymes share L-arginine as substrate, and many NOS inhibitors such as the L-arginine analogs *N*-nitro-L-arginine (L-NNA), nitro-L-arginine methyl ester, and *N*-monomethyl-L-arginine cannot distinguish the enzymatic isoforms. A number of inhibitors potentially selective for the NOS isoforms have been developed, but no known inhibitor is completely selective for one isoenzyme. Moreover, these compounds may have additional pharmacological effects unrelated to the NO pathway. Their effects are also hindered by variable bioavailability, and inhibition in particular tissues may not be complete. In addition, these agents are difficult to administer continuously in whole animals to mimic pathological states of chronic depletion.

Advent of molecular biology and stem cell technology allowed the specific deletion or "knockout" of genes in whole animals. This has been applied to generate mice lacking nNOS (8), eNOS (5, 9), and iNOS (13, 14, 26) (designated $nNOS^{-/-}$, $eNOS^{-/-}$, and $iNOS^{-/-}$). Such mice have become invaluable tools in defining the role of NO from various enzymatic sources in the gastrointestinal tract (Table 2). The purpose of this report is to summarize early lessons from available studies of the gastrointestinal tract using the NOS knockout animals.

NO IN NEUROTRANSMISSION AND GUT MOTILITY

The physiological role for NO as a nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter in the gut was first proposed by Bult and colleagues in 1990 (2). They showed that relaxation of the ileocolonic junction following electrical field stimulation of NANC nerves was suppressed by a blocker of NOS. The substance released during this stimulation resembled the vasodilator first described by Furchgott et al. (3a) as endothelium-derived relaxing factor. An ensuing

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Table 1. *NOS isoforms in the gastrointestinal tract*

Isoform	Expression	Activity	Tissue Distribution
nNOS (type I)	Constitutive	Ca^{2+} dependent	Neurons Skeletal muscle Neutrophils Pancreatic islets Smooth muscle Epithelium
eNOS (type III)	Constitutive	Ca^{2+} dependent	Endothelium Smooth muscle Neurons
iNOS (type II)	Inducible	Ca^{2+} independent	Macrophages Neurons Endothelium Other cell types

nNOS, neuronal nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS.

plethora of studies demonstrated that blockers of NO production suppress NANC nerve-mediated smooth muscle relaxation in various segments of the gut, thereby suggesting a role for NO in inhibitory transmission throughout the gut (23a).

These chemical blockers of NO production, however, could not identify the enzymatic source of NO in the inhibitory neurotransmission. Studies using NOS knockout animals showed that lower esophageal sphincter (LES) relaxation during electrical field stimulation of the NANC nerves was absent in $nNOS^{-/-}$ animals but was normal in $eNOS^{-/-}$ animals. This lack of LES relaxation was also noted in wild-type or $eNOS^{-/-}$ tissues treated with NOS inhibitors (12). These observations established that nNOS is the enzymatic source of NO in inhibitory neurotransmission in the LES.

Before the discovery of NO, ATP-like purine and the peptide vasoactive intestinal peptide (VIP) had been shown to be NANC-inhibitory neurotransmitters. Studies in mice lacking nNOS have clarified the role of NO among other inhibitory neurotransmitters in the gut. Besides mechanical responses of the muscle, several studies characterized the hyperpolarizing responses of the smooth muscle, called inhibitory junction potential (IJP), to NANC nerve stimulation (in the presence of adrenergic and cholinergic blockers). The wild-type animals showed a two-component IJP consisting of an apamin-sensitive, ATP-mediated fast IJP and an L-NNA-sensitive, NO-mediated slow IJP (15). $nNOS^{-/-}$ tissues showed only an apamin-sensitive, ATP-mediated fast IJP. Besides establishing the role of NO in inhibitory neurotransmission, these studies established a non-NOS-mediated and parallel purinergic-inhibitory neuromuscular transmission in the mouse stomach (15).

Table 2. *Phenotypes of NOS knockout mice*

Mutant Mice	Phenotype
$nNOS^{-/-}$	Gastroparesis, abnormal IJP and NANC relaxation in GI smooth muscle Resistance to ischemic and certain inflammatory injury Preserved hippocampal long-term potentiation
$eNOS^{-/-}$	Elevated systemic and pulmonary pressure Increased ischemic injury and certain inflammatory injury
$iNOS^{-/-}$	Increased susceptibility to bacterial and viral pathogens Increased susceptibility to tumors Resistance to sepsis-induced hypotension

IJP, inhibitory junction potential; NANC, nonadrenergic noncholinergic; GI, gastrointestinal.

Studies in $nNOS^{-/-}$ mice have also helped clarify the role of VIP. VIP had been shown to cause relaxation of gastric smooth muscle strips as well as of isolated smooth muscle cells. A large body of data showed that VIP acts directly on the smooth muscle cells via two separate VIP receptor types. Activation of these receptors leads to intracellular accumulation of cAMP as well as cGMP, both of which cause muscle relaxation. The cGMP accumulation in the smooth muscle cells is due to activation of NOS within the smooth muscle cells. Besides causing smooth muscle relaxation, NO produced in the smooth muscle was also thought to diffuse back and enhance release of VIP from the nerve terminals. Thus VIP was thought to be the main inhibitory neurotransmitter released from the prejunctional nerve endings, whereas NO was thought to enhance VIP release and mediate VIP action in the postjunctional smooth muscle cells (6). This role of NO as an intracellular mediator is quite different from its role as a neurotransmitter. To resolve this issue, the NOS isoform that was the source of NO in the action of VIP and cellular localization of that NOS isoform needed to be established. Studies have shown that eNOS is the main constitutive form of NOS in the postjunctional smooth muscle cells, whereas nNOS is primarily present in the prejunctional nerve endings (25). Studies using knockout animals showed that VIP hyperpolarized smooth muscles in the $eNOS^{-/-}$ in a manner similar to that in wild-type mice; however, VIP failed to produce hyperpolarization in $nNOS^{-/-}$ mice, thereby establishing that nNOS is the source of NO in the hyperpolarizing action of VIP. The role of VIP in the NO-mediated slow IJP had been established by showing that the nitrergic slow IJP was also suppressed by VIP receptor antagonists. Thus VIP released from nerve varicosities on stimulation acts as an autocrine mediator on the nerves to release NO from nNOS (Fig. 1). These conclusions are supported by immunohistochemical studies that colocalize nNOS and VIP, particularly in nerve varicosities of the myenteric neurons. If proven by further studies, this model of a serial role of VIP and NO during NANC nerve stimulation could provide a mechanism for the on-demand synthesis and release of NO, which, as a gas, cannot be stored in synaptic vesicles.

Besides mediating smooth muscle relaxation by causing smooth muscle membrane hyperpolarization (called electromechanical coupling), VIP may also cause relaxation without associated hyperpolarization (called pharmacomechanical coupling). NO-independent relaxation of the stomach due to VIP after vagal stimulation has been reported (24). It has been shown that VIP causes relaxation of the stomach in mice lacking cGMP kinase (18); this cGMP-independent relaxation appears to be mediated by cAMP. Further mechanical studies using $nNOS^{-/-}$ mice may help resolve the role of VIP in inhibitory neurotransmission, and studies using smooth

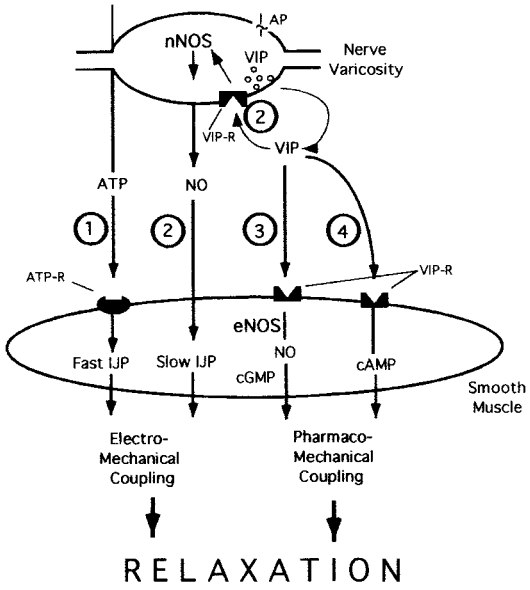

Fig. 1. Contribution of nitric oxide (NO) from neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) and its interactions with ATP and vasoactive intestinal peptide (VIP) in inhibitory neurotransmission revealed by the use of NOS knockout animals. On the basis of cumulative work of many investigators, ATP and VIP are two primary neurotransmitters released from nerve endings by an electrical action potential (AP). Together, the inhibitory transmission can be divided into four components. *1*) ATP-mediated, apamin-sensitive component: ATP stimulates P_2 receptor (ATP-R) on the smooth muscle to open apamin-sensitive potassium channels. This produces smooth muscle hyperpolarization, called fast inhibitory junction potential (IJP). *2*) VIP-mediated, nNOS-dependent component: VIP activates prejunctional VIP receptors (VIP-R) on prejunctional nerve endings and activates nNOS to cause synthesis and release of NO from nerve terminals. Diffused NO causes slow IJP in the postjunctional smooth muscle cells. *3*) VIP-mediated, eNOS-dependent component: VIP activates postjunctional receptors, possibly the natriuretic peptide clearance receptor, on smooth muscle cells to cause activation of membrane-bound eNOS to release NO within the smooth muscle. *4*) VIP-mediated, NOS-independent component: VIP activates postjunctional VIP-R on the smooth muscle cells to cause intracellular accumulation of cAMP. ATP- or VIP-mediated nNOS-dependent components involve smooth muscle membrane hyperpolarization and relaxation (electromechanical coupling). On the other hand, VIP released from nerves after stimulation may act directly on smooth muscle cells without producing hyperpolarization (pharmacomechanical coupling) by either increasing cAMP or by stimulating eNOS and thereby increasing cGMP in the smooth muscle cells. Further studies with NOS knockout mice are needed to resolve these pathways.

muscle cells from $eNOS^{-/-}$ mice may establish the possible role of VIP-stimulated NO from eNOS.

NO from eNOS may also be involved in retrograde neurotransmission in the central nervous system. On the basis of the blocking effects of L-NNA, NO was thought to be an essential retrograde messenger in postsynaptic neurons that feed back to presynaptic neurons for long-term potentiation (LTP). LTP is responsible for the persistent increase in activity of the presynaptic neurons and plays an important role in the process of learning and memory. Because the predominant form of NOS in neurons is nNOS, it was thought that nNOS was the source of NO in LTP. Surprisingly, LTP was not affected in $nNOS^{-/-}$ animals, suggesting that eNOS may be the source of NO. However, LTP was also unaffected in $eNOS^{-/-}$ animals. On the other hand, LTP was abolished in nNOS and eNOS double-knockout animals (22). These studies revealed that NO from both nNOS and eNOS is responsible for LTP.

Large amounts of NO from iNOS induced during inflammation may have major effects on gastrointestinal motility. For example, NO from iNOS induced by endotoxin and interleukin-1β produces gastric stasis and delays gastric emptying of solids (20). NO from iNOS may also be involved in the ileus. Thus, paradoxically, both increased NO from iNOS during inflammation and decreased NO as inhibitory neurotransmitter from nNOS result in gastric stasis. NO is a very labile molecule, known to exist in different redox states with different biological targets (23). The native form of NO involved in the action of NO may depend to some extent on the milieu and the distance between the sites of origin and the target (4). The neurotransmitter NO may exist as NO·, the endothelial-derived relaxing factor may exist as a nitrosothiol, and the inflammatory mediator may exist in various redox forms, including as a complex with other oxygen radicals. Further studies using the knockout mice would help test this hypothesis.

NO IN VASODILATION

NO donors have long been known to cause relaxation of vascular smooth muscle and vasodilation. NO from any of the NOS isoforms can be involved in the vasodilatory responses. Use of NOS knockout animals have helped distinguish the roles of NO from various NOS isoforms in intravascular pressure and flow under different physiological conditions.

The role of NO as endothelium-derived relaxing factor released in response to ACh and other agents is now well established. The main NOS isoform in the endothelium is eNOS. $eNOS^{-/-}$ mice have hypertension and lack a vasorelaxatory response to ACh (9). In concert with this finding, mice with transgenic overexpression of eNOS are hypotensive (17). NO from eNOS is an important regulator of local vasodilatory responses. This is demonstrated by the observation that $eNOS^{-/-}$ mice have a larger infarction size after cerebral ischemia.

Blood vessels are also under the control of excitatory and inhibitory nerves that cause vasoconstriction and vasodilation, respectively. NO from nerve endings containing nNOS is an important inhibitory neurotransmitter for vascular smooth muscle. Studies with knockout animals have established the role of both nNOS and eNOS in vasodilation. For example, pial vessel dilation induced by ACh is blocked by L-NNA and was assumed to be due to NO from NOS. However, both $nNOS^{-/-}$ and $eNOS^{-/-}$ animals showed normal vasodilation to ACh, which was blocked by L-NNA. In contrast to wild-type or $nNOS^{-/-}$ mice, the vasodilatory response in $eNOS^{-/-}$ mice was blocked by TTX, suggesting that both eNOS and nNOS are involved in the vasodilatory response in some systems (16). Penile vasodilation due to NO from inhibitory nerves was also initially thought to be the main factor in penile erection. However, studies showed

that penile erection was not affected in nNOS$^{-/-}$ animals, yet the erection in nNOS$^{-/-}$ mice was L-NNA sensitive (3). These observations suggest that NO from both nNOS and eNOS provide parallel pathways for vasodilation. Studies with double-knockout animals lacking both nNOS and eNOS may further clarify these issues. NO from iNOS also plays a role in vasodilation, particularly in pathological states, and is responsible for severe systemic hypotension in toxic shock (7).

NO IN INFLAMMATION AND INJURY

NO has been shown to play a complex role in augmenting or protecting tissue damage during inflammation. NO from different NOS isoforms may play different roles.

The role of NO from various isoforms of NOS using the knockout animals has been examined in trinitrobenzene (TNB)-induced colitis. nNOS$^{-/-}$ mice showed fewer inflammatory changes in TNB-induced colitis, suggesting that NO from NOS plays a detrimental role in TNB-induced colitis. On the other hand, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice showed more severe colitis, suggesting a normally protective role of NO from eNOS and iNOS in this inflammatory model. The mechanisms of these diverse roles of NO from various sources are not currently well defined. It has been postulated that NO from nNOS may be involved in enhanced superoxide generation in injury. The protective effect of NO from eNOS may be due to increased mucin production and maintenance of local blood flow, thus preventing bacterial invasion of the gut. NO from iNOS is usually produced in very large quantities and has a more generalized action compared with NO from constitutive nNOS and eNOS that yield NO in small quantities but at well-localized sites. NO from iNOS plays an anti-inflammatory role by suppressing bacterial infection, causing viral resistance, and enhancing leukocyte recruitment. More severe colitis in iNOS$^{-/-}$ mice treated with TNB was associated with greater myeloperoxidase activity and macroscopic ulcer index (19).

In another model of colitis, namely dextran sodium sulfate (DSS)-induced colitis, nNOS$^{-/-}$ mice showed more severe inflammatory changes, whereas eNOS$^{-/-}$ animals showed less weight loss and mortality and iNOS$^{-/-}$ mice still showed greater damage, including greater weight loss, hematocrit scores, diarrhea, and mortality (1). The reason why nNOS$^{-/-}$ and eNOS$^{-/-}$ mice show opposing effects in TNB- and DSS-induced colitis models is not clear. Further studies in knockout animals will help resolve these issues.

EFFECT OF TIMING AND DURATION OF NO DEPLETION

Experiments with NOS inhibitors have been generally acute experiments, lasting hours. The findings in acute experiments may be quite different from those of chronic depletion states. For example, anesthetic response was found to be enhanced in wild-type mice treated acutely with L-NNA, but there was no difference in the response of wild-type mice treated for 1 wk or of nNOS$^{-/-}$ mice compared with wild-type mice (11).

Another important distinction of acute experiments using NOS inhibitors from experiments involving genetically altered mice is that these mice lack NOS throughout embryonic development, which may lead to structural changes. For example, deficiency of eNOS may have developmental effects, such as limb reduction defects (5). So far, no overt neuronal changes have been documented for any NOS mutants, including nerve density or distribution in the gut, which is a surprise considering the many postulated roles of NO in neuronal development. NO also plays an important role in apoptosis and exerts mainly antiproliferative actions; thus chronic removal of NO may directly increase tissue mass, as seen in the stomach.

Conditional knockouts, such as those using inducible repressors and recombinases, may allow temporal and spatial specificity of NO function and thus circumvent potential changes in embryological development if induced in adult mice. However, such mutants are not yet available for the NOS isoforms, and their use in gastrointestinal physiology is yet to be proven. Such "second generation" knockouts are not without their own perils. For example, promoters in these constructs have variable efficiency of induction in different tissues.

Another potential difference between acute experiments and chronic deficiency or excess of NO is the occurrence of changes in other mediators and processes that may induce compensatory increases in other mediators or functions, and chronic deficiency may reveal pathological consequences of life-long deficiency.

COMPENSATORY PHENOMENA ASSOCIATED WITH NOS DEFICIENCY

Compensation for a missing enzyme is common in biology. This may occur via a normally present redundant pathway with functions similar to the missing enzyme. Examples of redundant parallel pathways involving other isoforms of NOS were described earlier for pial vasodilation, penile erection, and LTP in brain neurons.

Various NOS isoforms also have splice variants that may provide redundant parallel pathways to the functions of the main enzyme. It has been shown that, in the brain of nNOS$^{-/-}$ mice, levels of NOS catalytic activity are persistently low ($<5\%$). This residual activity is attributable to several alternatively spliced isoforms of nNOS that the mutated nNOS gene is still capable of producing in nNOS$^{-/-}$ mice. The existence of such alternatively spliced variants was not known during construction of the original targeting vector for nNOS$^{-/-}$ mouse. This nNOS$^{-/-}$ mouse may also express other potential splice variants with retained catalytic activity in the gut (10), which may mask the effect of nNOS deficiency.

Other mediators, such as the related gas carbon monoxide (CO), may play a parallel role as neural messenger. The role for CO as neurotransmitter was

suggested by altered intestinal motility in mice harboring a genomic deletion of the enzyme responsible for its biosynthesis, heme oxygenase-2 (HO-2). Both HO-2-deficient and nNOS$^{-/-}$ mice had diminished relaxation and decreased cGMP production on electrical field stimulation of the ileum under NANC conditions. However, nNOS$^{-/-}$ mice treated with the HO-2 antagonist SnPP-IX and HO-2-deficient mice treated with L-NNA showed almost complete abolition of relaxation and of cGMP production, suggesting that NO and CO have a parallel role in electrical field stimulation-induced relaxation in the ileum and together account for most of NANC-mediated relaxation (21). Other inhibitory neurotransmitters such as ATP, VIP, and pituitary adenylate cyclase-activating polypeptide provide important inhibitory pathways in certain tissues and may also compensate for the loss in NO inhibitory neurotransmission.

TISSUE-SPECIFIC MANIFESTATION OF NOS DEFICIENCY

Although NO from nNOS has been shown to be an inhibitory neurotransmitter throughout the gut, all organs are not equally affected in nNOS-deficient animals. This may be due to differences in parallel redundant pathways, in the NOS isoforms, in expression of alternatively spliced isoforms of a particular NOS, and in compensation by other neurotransmitters and chemicals. For example, NO deficiency produces nearly complete loss of nerve-stimulated smooth muscle relaxation in the LES and stomach. However, in the small bowel, chemical blockers of NOS or nNOS$^{-/-}$ animals have only partially suppressed relaxation following NANC nerve stimulation.

The splice variants of nNOS are prominently expressed in the small intestine and colon but not in the stomach. These splice variants are preserved in the nNOS-deficient mice. This difference may explain why smooth muscle hypertrophy and dilation are marked in the stomach and duodenum, but less marked changes are observed in the small and large intestines of nNOS$^{-/-}$ mice (10). Further studies are needed to test this possibility. Alternate splicing of iNOS also generates splice isoforms, but their tissue expression and function are not known. Other factors may also explain differences in the phenotype of stomach vs. small and large bowel in nNOS$^{-/-}$ mice. HO-2 is highly expressed in the small bowel but not in the stomach. Similarly, purinergic inhibitory neurotransmission is more marked in the small bowel but appears less marked in the stomach and is least obvious in the esophagus.

SUMMARY

In summary, the use of NOS gene knockout animals has helped in elucidating the roles of NO in inhibitory neurotransmission, regional blood flow, and inflammation in the gut from different NOS isoforms. These animals have helped resolve some of the complexities of the NO signaling system, the redundancies and compensations brought into play in the absence of the individual enzymatic isoforms, and the phenotype of lifelong deficiency of these enzymes. These early results demonstrate the enormous potential of genetically engineered mice lacking specific genes in elucidation of physiology and pathophysiology.

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