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Review

Cardiovascular roles of nitric oxide: A review of insights from nitric oxide synthase gene disrupted mice†

Victor W.T. Liu and Paul L. Huang*

Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, United States

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Nitric oxide (NO) is a gaseous molecule that plays many key roles in the cardiovascular system. Each of the enzymes that generate NO—neuronal, inducible and endothelial NO synthase—has been genetically disrupted in mice. This review discusses the cardiovascular phenotypes of each of the NO synthase (NOS) gene knockout mice, and the insights gained into the roles of NO in the cardiovascular system. Mice lacking the endothelial isoform are hypertensive, have endothelial dysfunction and show a more severe outcome in response to vascular injury, to stroke and cerebral ischaemia, and to diet-induced atherosclerosis. Mice lacking the neuronal isoform show a less severe outcome in response to stroke and cerebral ischaemia but have increased diet-induced atherosclerosis. Mice lacking the inducible isoform show reduced hypotension to septic shock. Together, NOS gene knockout mice have been useful tools that complement our other approaches to studying the multiple roles of NO in the cardiovascular system.

1. Introduction

Nitric oxide (NO) is a key signaling messenger in the cardiovascular system.[1] In addition to its role as endothelium-derived relaxing factor (EDRF), NO serves many important biological functions in cardiovascular physiology. NO maintains vascular integrity by inhibiting platelet aggregation,[2,3] leukocyte–endothelium adhesion[4–6] and vascular smooth muscle proliferation.[7] In addition, NO is produced in cardiac smooth muscle, where it regulates cardiac contractility.[8] Adequate levels of endothelial NO are important to preserve normal vascular physiology—in the face of diminished NO bioavailability, there is endothelial dysfunction, leading to increased susceptibility to atherosclerotic disease.[9–11] Atherosclerosis, hypertension, hypercholesterolemia, diabetes mellitus, congestive heart failure, thrombosis and stroke have all been linked to abnormalities in NO signaling.[10,12,13] Genetic manipulation of the enzymes that generate NO in mice has contributed significantly to our understanding of its many roles, both in physiology and in disease pathogenesis.

2. Nitric oxide synthases

NO is produced by nitric oxide synthase (NOS) enzymes, of which there are three main isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS).[14] *Table 1* shows a comparison of these isoforms. The three NOS isoforms are encoded on separate chromosomes by separate genes. They share homology in regions involved in cofactor binding (for example, FAD, FMN, and NADPH ribose and adenine binding sites), and have similar enzymatic mechanisms that involve electron transfer for oxidation of the terminal guanidino nitrogen of L-arginine. However, their expression patterns differ, as do the detailed regulations of their activity. nNOS is predominantly expressed in certain neurons and in skeletal muscle, whereas eNOS is predominantly expressed in endothelial cells. iNOS is expressed by macrophages and cells of macrophage/monocyte lineage. Despite their names, a variety of cell types express these isoforms, with many tissues expressing more than one isoform. Furthermore, the innervation and vasculature in all tissues have the potential to express nNOS and eNOS, while circulating blood elements may express iNOS.

Both nNOS and eNOS are generally constitutively expressed; their activities are primarily regulated by intracellular Ca^{2+}/calmodulin levels. In contrast, iNOS expression is induced in activated macrophages as an immune response. For enzymatic activity, NOS proteins must bind cofactors and dimerize.[14] NOS proteins first bind to the cofactors FAD and FMN. The additions of L-arginine, BH_4 and heme allow the NOS protein to form dimers. eNOS and nNOS dimers formed this way are inactive, and depend on calmodulin binding stimulated by increases in intracellular calcium. In contrast, the iNOS dimers bind calcium/calmodulin and are

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* Corresponding author. Cardiovascular Research Center, Massachusetts General Hospital East, 149 Thirteenth Street, Charlestown, MA 02129. Tel: +1 617 724 9849; fax: +1 617 726 5806.
E-mail address: phuang1@partners.org (P.L. Huang).

Table 1 Characteristics of NOS Isoforms

Isoform	nNOS	iNOS	eNOS
Other names	NOS-1, NOSI, Type I NOS	NOS-2, NOSII, Type II NOS	NOS-3, NOSIII, Type III NOS
Human chromosomal location	12q24.2-12q24.3	17cen-q11.2	7q35–7q36
Human gene structure and size	29 exons locus region >200 kbp	26 exons 37 kbp	26 exons 21–22 kbp
Human monomer size (predominant form)	161 kDa	131 kDa	133 kDa
Splice variants	Yes	Yes	No
Typical site of expression	Neurons	Macrophages	Endothelial cells
Other major sites of expression	Smooth muscle Skeletal muscle	Smooth muscle Liver	Smooth muscle Platelets
Gene expression	Constitutive and inducible	Inducible	Constitutive and inducible
Ca^{2+} dependency	Ca^{2+}-dependent	Practically Ca^{2+}-independent	Ca^{2+}-dependent
Covalent modifications	Phosphorylation		Myristoylation Palmitoylation Phosphorylation
Protein–protein interactions	hsp90, caveolin, NOSIP		hsp90, caveolin
Subcellular localization	Neuromuscular junction Soluble Sarcoplasmic reticulum	Soluble	Caveolae
Means of localization	N-terminal PDZ domain (for membrane association)	N/A	N-terminal myristoylation
NO production (range)	Moderate (nM to μM)	High (μM)	Low (pm to nM)

active even at low (resting intracellular) concentrations of calcium. Thus, the main switch for activity for nNOS and eNOS is a transient increase in intracellular calcium concentration, whereas the main switch for iNOS is at the level of transcription.

The DNA and protein sequences of the NOS isoforms are conserved between species, with nNOS, eNOS and iNOS sharing up to 96%, 93% and 80% amino acid sequence identity between mice and humans. Within each species, the NOS isoforms share about 51–59% amino acid sequence identity. Each isoform has notable structural features. The nNOS gene encodes a PDZ domain in exon 2 that is required for membrane association.[15] Several nNOS splice variants lack exon 2, resulting in expression of cytoplasmic nNOS that lacks subcellular localization sequences.[16,17] In endothelial cells, eNOS is localized to caveolae by N-terminal fatty acid modifications—myristoylation and palmitoylation,[18–21] as well as interactions with heat shock protein hsp90 and caveolins.[12,22] Caveolins (caveolin-1 in endothelial cells and caveolin-3 in cardiac muscle) bind to eNOS and inhibit its activity. eNOS is also regulated by phosphorylation at multiple sites, including serine 1179 and threonine 497.

In addition to nNOS, eNOS, and iNOS, there is a constitutively active NOS isoform present in mitochondria, referred to as mtNOS.[23,24] mtNOS is located in the inner mitochondrial membrane, and likely plays key roles in modulating mitochondrial respiration and mitochondrial transmembrane potential. However, whether mtNOS corresponds to one of the three known isoforms is not known.

3. Molecular targets of NO

In many cells and for many of the biological signaling roles of NO, the physiologic target is soluble guanylate cyclase.[25,26] NO activates guanylate cyclase by binding to its heme moiety, resulting in increased cGMP levels. This is responsible for events in the brain following NMDA receptor activation. Garthwaite first described that cultures of cerebellar cells produce cGMP in response to the excitatory amino acid neurotransmitter glutamate.[27] In the vasculature, cGMP mediates NO-dependent relaxation of vascular smooth muscle, resulting in vasodilation. Similarly, NO produced as a neurotransmitter in the autonomic nervous system innervating the gastrointestinal tract, urinary tract, and the respiratory tract, mediates smooth muscle relaxation in these tissues by increases in cGMP production. These effects are likely mediated by the phosphorylation of downstream proteins by cGMP-dependent protein kinases, including myosin light chain.

Another target for NO is sulfhydryl groups on proteins, to form nitrosothiol compounds.[28] One such protein is hemoglobin, which may serve as a natural carrier for NO.[29] In cardiac muscle, NO S-nitrosylates the ryanodine receptor on the sarcolemmal membrane and is required for its normal activity.[30] NO also nitrosylates critical residues in *N*-ethylmaleimide-sensitive factor, which is important to the regulation of exocytosis.[31] NO can react with superoxide anion to form peroxynitrite anion.[32–34] Large quantities of NO, made for instance by iNOS, also can directly inhibit mitochondrial complexes I and IV.[35–37] Finally, NO can activate the enzyme poly-ADP ribose polymerase (PARP), resulting in depletion of cellular energy stores.[38–40] Generally, these latter mechanisms underlie some of the toxicity of NO, while effects on soluble guanylate cyclase and S-nitrosylation of proteins mediate many of the biological signaling roles of NOS. In addition to NO, NOS enzymes are capable of generating reactive oxygen species. When there is insufficient BH_4, electron transfer in eNOS becomes 'uncoupled,' so the enzyme produces superoxide (O_2^-). This superoxide may react with NO to form peroxynitrite ($ONOO^-$). Under conditions of limited L-arginine bioavailability, eNOS may generate H_2O_2.[41]

Table 2 Functions of NOS Isoforms and Phenotypes of NOS Knockout Mice[139]

Isoform	Function	Phenotype of Knockout Mice
nNOS	Signal transduction	Enlarged stomach, pyloric stenosis
	Neurotransmission	Normal CNS development
	Toxicity (at high levels)	Aggressive behavior
		Decreased neuronal injury after stroke (acute)
		No protection from rapid cerebral ischaemic preconditioning
		Increased diet-induced atherosclerosis
		Reduced cardiac contractile response to β-adrenergic stimulation
iNOS	Defense against pathogens	Susceptible to tuberculosis & other infections
	Inflammation	Reduced hypotension in sepsis
		Decreased neuronal injury after stroke (delayed)
		Decreased diet-induced atherosclerosis
eNOS	Vasodilation	Hypertension
	Modulation of platelet aggregation	Absence of EDRF
	Modulation of leukocyte–endothelial interactions	Increased vascular response to injury
		Increased neuronal injury after stroke (acute)
		No protection from rapid cerebral ischaemic preconditioning
		Increased diet-induced atherosclerosis
		Enhanced cardiac contractile response to β-adrenergic stimulation

4. Genetic approaches

NOS enzymes can be inhibited by pharmacologic agents, including arginine analogs substituted at the terminal guanidino nitrogens. These arginine analogs bind to NOS, but cannot serve as substrate, so they compete with L-arginine and inhibit the enzyme. Such pharmacologic NOS inhibitors have yielded a tremendous amount of valuable information. Indeed, blockade of a biological process by L-nitro-arginine (L-NA) or L-*N*-arginine-methyl-ester (L-NAME), and outcompetition of this effect by an excess of L-arginine, provides very strong evidence for the involvement of NO in that process. One potential limitation of pharmacologic inhibitors, however, is that they may inhibit more than one NOS isoform. There are also structurally distinct inhibitors of NOS that are not arginine analogs (e.g. 7-nitroindazole or aminoguanidine), but some these agents have unrelated effects as well.

A complementary approach is to manipulate the genes that encode the NOS enzymes to generate knockout mice in which a particular NOS gene has been disrupted. This approach complements pharmacologic approaches because its specificity is at the genetic level. It pinpoints the roles of individual NOS genes, since many tissues contain all three of the major NOS isoforms. Further, it allows the study of how chronic absence of the NOS isoform affects physiology in intact animals. *Table 2* shows some of the functions of the NOS isoforms, and the phenotypes of NOS knockout mice.

Several issues unique to the genetic approach should be kept in mind, as they can potentially confound studies using knockout animals. First, there may be developmental abnormalities due to the gene knockout. If one of the NOS isoforms plays a critical role in embryonic development, its absence may lead to other secondary abnormalities that are difficult to predict. Second, additional phenotypes may emerge from changes to pathways that act upstream or downstream to the gene product of interest. Third, other isoforms or gene products, acting in parallel, may compensate for the absent gene product and mask possible phenotypes. Finally, embryonic stem cells used to generate knockout mice are often derived from particular strains like the SV129 strain that are better sources of pluripotent embryonic stem cells. These knockout mice have mixed genetic background, which may itself lead to phenotypic abnormalities. For this reason, the knockout mice are usually backcrossed for 10 generations, often to the standard C57BL/6 strain, to minimize the effects of genetic background variation.

5. nNOS knockout mice

The first line of nNOS knockout mice was established by disrupting exon 2 of nNOS using homologous recombination.[42] This region contains the sequence for the ATG initiation codon and the PDZ domain that is responsible for membrane association.[16] Therefore, these mutant mice do not express nNOSα, the predominant splice form of nNOS, as detected by Western blot analysis or NADPH diaphorase staining. They have significantly diminished NO production in the brain, as measured by NOS enzymatic assay, cGMP levels, and measurement of NO by spin trapping.[43–46] nNOS splice variants (nNOSβ, nNOSγ, and the testicular isoforms) that lack exon 2 are still expressed and account for less than 5% of all nNOS catalytic activity in the brain. These splice variants are soluble, since they lack the PDZ domain.

The most apparent phenotype of nNOS knockout mice is enlargement of the stomachs, often to several times the normal size, demonstrating a role for nNOS in smooth muscle relaxation of the pyloric sphincter. nNOS knockout mice are also resistant to focal and global cerebral ischaemia, consistent with a role for nNOS-derived NO in cellular injury following ischaemia.[46–49] These mice are fertile and viable, although male mutants are more aggressive than their wild-type littermates.[42,50] A separate line of nNOS knockout mice was established by ablating exon 6, which encodes the heme-binding domain of nNOS required for

catalytic activity.[51] These mice have a more severe pyloric stenosis phenotype and also reproductive endocrine abnormalities. [^{3}H]citrulline measurements indicate that nNOS activity in exon 6-deficient mice is 0.3% of that in wild-type, compared to 5% in exon 2-deficient mice.

6. eNOS knockout mice

The first eNOS knockout mice were generated by disrupting the region that encodes for the NADPH ribose and adenine binding sites, which are essential for catalytic activity.[52] These mice are viable, fertile and exhibit no gross anatomic abnormalities, despite the absence of detectable eNOS mRNA, protein or enzymatic activity. As outlined below, eNOS knockout mice show abnormalities in vascular relaxation, blood pressure regulation, and cardiac contractility. They are a useful animal model for endothelial dysfunction, as they show increased propensity to form neointima in response to vessel injury,[53,54] and accelerated and more severe diet-induced atherosclerosis in the apolipoprotein E (apoE) knockout mouse model.[55,56]

Several additional strains of eNOS knockout mice have also been reported. In one strain, the eNOS gene was disrupted at the calmodulin binding site, encoded by exons 12 and 13,[57] In another strain, the NADPH ribose and adenine binding sites were disrupted,[58] similar to the first eNOS knockout mice.[52] All three known eNOS knockout mice have similar phenotypes with hypertension and vascular abnormalities. The fact that independently generated mice, particularly those in which different parts of the eNOS gene were targeted, have similar phenotypes, adds confidence that the observed phenotypes are specific.

7. iNOS knockout mice

Three separate groups independently disrupted the iNOS gene. MacMicking *et al*. deleted the promoter region and the first four exons, including the initiation codon ATG.[59] Wei *et al*. in an attempt to delete the first five exons of the gene, created a genomic rearrangement of the iNOS gene that results in an aberrant transcript, but no detectable iNOS activity.[60] Laubach *et al*. disrupted the calmodulin, FAD, and FMN binding domains of iNOS, and found no detectable iNOS mRNA or protein.[61] In all cases, expression of iNOS cannot be induced in the iNOS mutant mice under conditions that induce iNOS in wild-type animals. Peritoneal macrophages from all of the iNOS mutant mice are deficient in NO and nitrite production. None of the mutants demonstrate abnormalities in growth, fertility, or gross histopathology.

Initial characterization of these iNOS mutant animals centered on two proposed functions of iNOS: cell-mediated resistance to pathogens, and hemodynamic responses to septic shock. Inducible NOS mutant mice are more sensitive to the intracellular pathogen *Listeria monocytogenes*[59] and to the intracellular protozoan parasite *Leishmania major*[60] than are wild-type mice. Both are pathogens that elicit cell-mediated immune responses, and the increased susceptibility of iNOS mutant mice demonstrates the importance of iNOS to host defenses against these pathogens.

The role of iNOS in septic shock is supported by the finding that NO is produced in large quantities during infection.[62] Inappropriate vasodilation, abnormal regulation of blood flow to organs, myocardial suppression, and interference with cellular respiration all contribute to hypotension and mortality in septic shock. Multiple studies show that NOS inhibitors can reverse the hypotension of patients in septic shock,[63,64] or of animals treated with LPS or TNF.[65,66] iNOS mutant mice have a blunted hypotensive response to sepsis and LPS.[59–61] These findings suggest that NO generated by iNOS contributes to the inappropriate vasodilation and hypotension seen in sepsis.

8. Endothelium-derived relaxing factor activity

In 1980, Furchgott and Zawadzki found that acetylcholine is able to cause relaxation of blood vessels if, and only if, the endothelium is intact. This indicated that acetylcholine does not act directly on vascular smooth muscle, but rather, that the endothelium plays a key role in vasodilation. This led to the proposal of the existence of endothelium-derived relaxing factor, or EDRF.[67] Subsequent pioneering work led to the identification of EDRF as nitric oxide.[68–71] Pharmacologic inhibitors of NOS such as L-NA and L-NAME also abrogate vasodilation to acetylcholine, and in their presence, acetylcholine actually causes a slight increase in vascular tone.

One of the first experiments in eNOS knockout mice was to replicate the experiments of Furchgott and Zawadzki. In fact, isolated aortic rings from eNOS knockout do not respond to acetylcholine in organ baths.[52] These aortic rings do respond to the exogenous NO donor sodium nitroprusside and to papaverine, indicating that the vascular smooth muscle is capable of relaxation. These observations establish that eNOS is an essential source of NO in the vasculature, and it is required for EDRF activity.

9. Blood pressure

There are several interacting homeostatic regulators of blood pressure, including the renin–angiotensin system, the autonomic nervous system, and local mediators such as EDRF.

L-NA and other NOS inhibitors cause a rise in blood pressure in many species, including rats, guinea pigs, rabbits, dogs and mice.[72] This effect is consistent with the predicted role for basal NO production in the regulation of blood pressure. Therefore, it was of particular interest to examine basal blood pressure in the eNOS mutant mice to see if other homeostatic mechanisms would compensate for the absence of endothelial NO production.

The blood pressure of eNOS knockout mice is about 30% higher than that of wild-type animals. This is true regardless of the types of anesthesia used (and in the awake state), and is also true for independently generated eNOS knockout mouse strains. Thus, eNOS plays a key role in regulation of blood pressure. However, it is not clear why other homeostatic systems cannot compensate for absence of eNOS. The set point for systemic blood pressure is regulated through integration of cardiac, neuronal, humoral and vascular mechanisms. One possibility is that the renin–angiotensin system and autonomic nervous system evolved to serve primarily as a defense against hypotension, and diminution in their activity is a poor buffer against hypertension. Alternatively, eNOS (and indeed other NOS isoforms) may be involved in establishing the baroreceptor set point.[73,74]

eNOS mutant mice show a decrease in blood pressure in response to L-NA. This hypotensive effect of L-NA is prevented by L-arginine and is not observed with D-nitro-arginine. This suggests that non-endothelial NOS isoforms may play a direct or indirect role in the maintenance of blood pressure. nNOS knockout mice, whose blood pressure values are similar to wild-type littermates when awake, tend to be hypotensive under anesthesia.[45,75] These results suggest an opposite role for nNOS, in raising or maintaining vascular tone. nNOS is present in central vasomotor centers, perivascular nerves, and skeletal muscle, and its effects in these locations may counter the direct vasodilatory effect of NO in vessels. Multiple roles for endothelial and non-endothelial NOS isoforms in vasodilation and vasoconstriction could also explain the observed variability in maximal pressor effects of various NOS inhibitors.

10. Vascular function and dysfunction

NO is critical to the pathophysiology of vascular disease and the concept of endothelial dysfunction. Endothelial dysfunction is defined as impairment of physiologic endothelium-dependent relaxation. It occurs in atherosclerosis, hypertension, diabetes, hypercholesterolemia, and normal aging.[9–11] Impairment of endothelial function occurs before structural changes such as intimal hyperplasia or lipid deposition. This is therefore an early event in the pathophysiology of atherosclerosis. Clinically, endothelial function can be tested by using ultrasound to determine the forearm blood flow response to reflow hyperemia. Experimentally, endothelial function can be tested by using a myograph to determine the vasodilator response of an isolated vessel segment to pharmacologic agents such as acetylcholine, bradykinin, and VEGF. Endothelial dysfunction is characterized by diminished endothelial NO levels. Because eNOS knockout mice completely lack endothelial NO production, they serve as a model of extreme endothelial dysfunction.

There are several potential mechanisms for endothelial dysfunction, as outlined in *Figure 1*.[76] These can be separated into three broad categories: reduced eNOS expression levels, reduced eNOS enzymatic activity, and rapid removal of NO. First, changes in eNOS mRNA or protein expression levels can lead to a reduction in eNOS activity.[77] However, most evidence from animal models and humans suggests there is an increase in the amount of eNOS with diabetes and atherosclerosis, rather than a decrease. Second, L-arginine, the substrate for NO production, can be limiting in tissues. An endogenous competitive inhibitor, asymmetric dimethylarginine (ADMA) may reduce endothelial NO production even in the presence of adequate L-arginine levels.[78,79] Third, eNOS requires FAD, FMN, NADPH, and BH_4 as cofactors. BH_4, whose synthesis is rate-limited by GTP cyclohydrolase, is a particularly important cofactor, because in its absence, electron transport through eNOS can become 'uncoupled,' resulting in generation of superoxide anion.[80] Fourth, eNOS requires dimerization and proper intracellular localization to caveolae, mediated in part by interactions with caveolin and hsp90.[12,22] Fifth, eNOS is phosphorylated at S1179 by Akt kinase and other kinases.[81,82] Sixth, NO produced by eNOS may be rapidly inactivated by reaction with superoxide (O_2^-) to form peroxynitrite anion ($OONO^-$).[32] This superoxide can be formed by NADPH oxidase,[83] or uncoupled eNOS.[80] These conditions may all contribute to endothelial dysfunction.

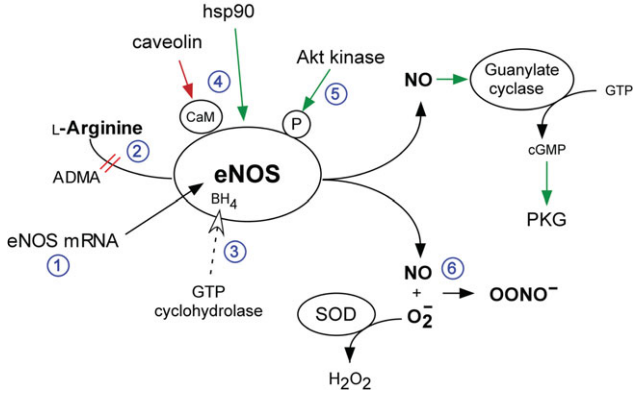

Figure 1 Regulation of eNOS activity and mechanisms for endothelial dysfunction. Several mechanisms can account for endothelial dysfunction, including: (1) changes in eNOS mRNA or protein levels; (2) decreased substrate availability; (3) decreased cofactor availability; (4) improper subcellular localization; (5) abnormal phosphorylation; and (6) scavenging of NO by superoxide (O_2^-) to form peroxynitrite anion ($ONOO^-$). ADMA, asymmetric dimethylarginine; SOD, superoxide dismutase; PKG, protein kinase G.

While eNOS plays important roles in vessel function, excessive NO production may contribute to the development of atherosclerosis. iNOS is expressed in activated monocytes and macrophages, and both iNOS and nNOS are expressed in vascular smooth muscle cells in atherosclerotic lesions.[84–86] These isoforms may contribute to lesion formation by forming NO which can react with superoxide to form the extremely potent oxidant peroxynitrite. NO and peroxynitrite can both increase oxidative stress and oxidize LDL.[87] Human atherosclerotic lesions contain nitrotyrosine, which is evidence for the presence of peroxynitrite. NO can also affect redox-sensitive transcription of genes involved in endothelial cell activation such as VCAM-1.[88,89]

11. Vascular injury and diet-induced atherosclerosis

Atherosclerosis is driven by biochemical, cellular, and hemodynamic forces in the vessel wall that cause vascular injury, ultimately leading to endothelial dysfunction, cellular proliferation, recruitment of inflammatory cells, and accumulation of oxidized LDL.[90] The response of blood vessels to injury is formation of neointima. Vascular smooth muscle cells proliferate in the medial layer and migrate across the internal elastic lamina to form the neointima. NO suppresses smooth muscle proliferation in response to vessel injury,[91] suggesting that it normally serves a protective role. In association with other effects such as inhibition of platelet aggregation and adhesion[2] and inhibition of leukocyte activation and adhesion,[5,92] NO normally suppresses the processes that lead to the development of atherosclerotic plaques. A relative deficiency in vascular NO would reduce these normally protective effects and thereby predispose to atherosclerosis.

To assess whether eNOS has a role in neointima formation following vascular injury, eNOS knockout mice were

subjected to a cuff model of vascular injury.[53] eNOS knockout mice show significantly greater neointima formation after cuff injury than wild-type mice. Thus, results from eNOS knockout mice confirm results using pharmacologic agents, and show that a deficiency in the amount of available NO in the vessel wall by itself increases neointimal formation in response to vascular injury.

To mimic human diet-induced atherosclerosis, apoE knockout mice have been a useful mouse model. apoE knockout mice develop spontaneous atherosclerotic lesions in their aortas which are exacerbated by a high fat, 'Western' diet.[93,94] To study the effects of individual NOS isoforms on diet-induced atherosclerosis, each NOS knockout mouse was bred onto the apoE knockout background, creating double knockout mice.

apoE/eNOS double knockout mice on a Western diet develop atherosclerosis significantly faster than, and have almost twice the atherosclerotic lesion areas as, apoE knockout mice on the same diet.[56] apoE/eNOS double knockout mice also show evidence of coronary artery disease, left ventricular dysfunction, aortic aneurysm and aortic dissection. The phenotype of apoE/eNOS double knockout mice more closely resembles the spectrum of cardiovascular complications seen in human atherosclerosis. It is also the first murine model to demonstrate spontaneous distal coronary arteriosclerosis associated with left ventricular dysfunction. These findings support the concept that restoration of eNOS function in patients with atherosclerosis is an important therapeutic goal. apoE/eNOS double knockout mice are hypertensive, but pharmacological control of blood pressure still leads to accelerated atherosclerosis and the development of aortic aneurysms.[55] Thus, the effects due to eNOS deficiency are not merely caused by hypertension.

In contrast, apoE/iNOS double knockout mice show significantly smaller lesion areas compared to apoE knockout mice at 16 and 24 weeks.[95] The lipoprotein profile, as assessed by FPLC, do not differ between apoE knockout mice and apoE/iNOS double knockout mice. The reduction in atherosclerosis in double knockout animals is associated with decreased plasma levels of lipoperoxides, suggesting that reduction in iNOS-mediated oxidative stress may explain the protection from lesion formation in double knockout animals. Thus, genetic deficiency of iNOS decreases atherosclerosis in Western diet-fed apoE knockout animals.

Like the apoE/eNOS double knockout mice, apoE/nNOS double knockout mice on a Western diet develop greater atherosclerotic lesion areas than apoE knockout mice.[96] RT-PCR shows that the predominant nNOSα splice variant is absent, although nNOSγ is present. nNOS deficiency significantly reduces the mean arterial blood pressure in female apoE knockout mice but is unchanged in male mice. apoE/nNOS double knockout mice also have higher mortality rates than apoE knockout mice. Thus, there is evidence that nNOS may serve atheroprotective roles, like eNOS.

12. Cerebral ischaemia

Following cerebral ischaemia, NO levels in the brain rise several orders of magnitude, from baseline nanomolar levels to stimulated micromolar levels.[97] nNOS knockout mice reveal that the nNOS isoform mediates this increase, since the knockout mice do not show it.[48] When subjected to a middle cerebral artery (MCA) occlusion model of focal ischaemia, nNOS knockout mice develop significantly smaller infarct sizes and have better neurological outcome than wild-type mice. These results confirm that although it normally serves important vascular physiological roles, NO overproduction in the setting of cerebral ischaemia actually contributes to tissue damage.

Measurements of regional cerebral blood flow (rCBF) by laser Doppler flowmetry show that both nNOS knockout mice and wild-type mice have similar reductions in blood flow. Thus, the smaller infarct sizes in the nNOS knockout mice cannot be explained by differences in cerebral blood flow.[48] Protection in nNOS knockout mice is also observed in transient and permanent focal ischaemia models and global ischaemia models.[47,49] The iNOS isoform is not present in ischaemic brain until days later, as glia and inflammatory cells enter the infarct zone. Like nNOS, iNOS contributes to tissue damage after cerebral ischaemia. Inhibition of iNOS by selective pharmacologic inhibitors, or gene deletion of iNOS, reduces this late damage.[98–100] nNOS and iNOS may contribute to neuronal toxicity in several ways, including reaction with superoxide to form peroxynitrite anion or otherwise increasing reactive oxygen species,[101] activation of poly-ADP ribose synthase resulting in depletion of cellular energy stores,[40] and direct inhibition of mitochondrial complexes I and IV.[102]

In contrast, eNOS knockout mice subjected to the MCA occlusion model develop larger infarct sizes compared to wild-type mice.[103] Laser Doppler flowmetry[103] and temporal correlation mapping[104] shows that eNOS knockout mice have significantly reduced blood flow than do wild-type mice. This confirms that eNOS normally serves to vasodilate and preserve blood flow in the setting of ischaemia; in its absence, inability to preserve blood flow contributes to the enlarged infarct sizes observed in eNOS knockout mice.

13. Ischaemic preconditioning

Ischaemic preconditioning (IPC) refers to processes by which brief, sublethal episodes of ischaemia stimulate a protective response against subsequent, more severe, ischaemia.[105] IPC has been described in many tissues, including the heart, brain, liver, and gastrointestinal tract. Although there are similarities between different tissues, it is not known whether the triggers and mediators of IPC are the same in all tissues. The potential protective mechanisms include alterations in cell death genes,[106] heat shock proteins,[107] lipid peroxidation,[108] inflammation,[109] and mitochondrial metabolism.[110] IPC has been divided into rapid and delayed forms. Rapid IPC occurs when the preconditioning stimulus precedes the severe ischaemic insult by a short time interval (minutes to several hours), while delayed IPC occurs requires a longer time interval (hours to days) to develop. It has been hypothesized that delayed IPC may involve changes in gene expression and new protein synthesis.

Pharmacologic studies suggest separable roles for each of the NOS isoforms in cerebral IPC. In a newborn rat model of hypoxia-ischaemia, preconditioning by mild hypoxia protects against more severe hypoxia 24 h later.[111] IPC protection is prevented by L-NA, but not the iNOS-specific inhibitor 7-nitroindazole or the iNOS specific inhibitor aminoguanidine, suggesting by process of elimination, involvement of

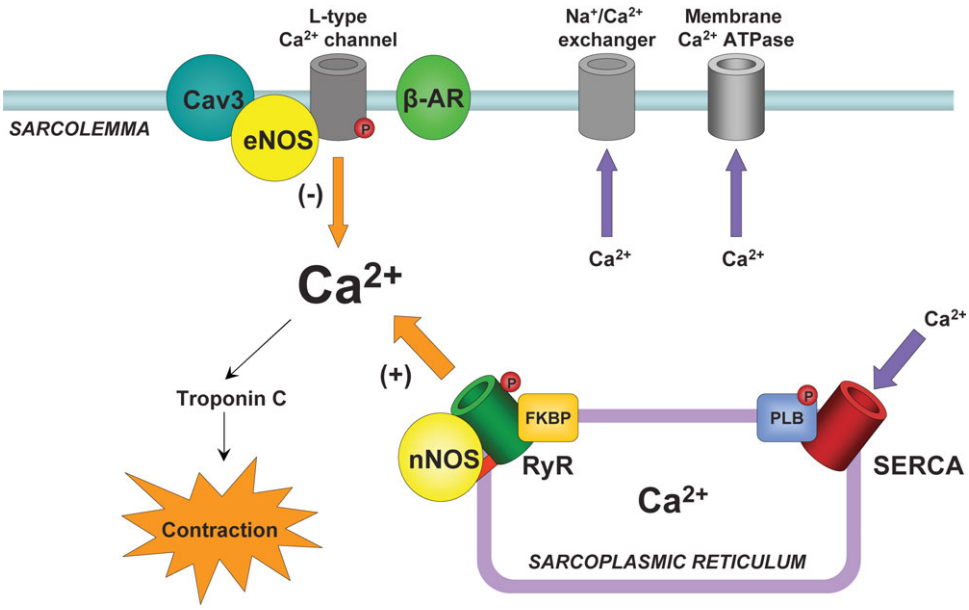

Figure 2 eNOS and nNOS in cardiac excitation–contraction coupling. In cardiac myocytes, eNOS associates with caveolin-3 at the sarcolemma, where it blunts inotropic response to isoproterenol stimulation. nNOS associates with the ryanodine receptor at the sarcoplasmic reticulum and is required for proper Ca^{2+}-mediated Ca^{2+} release. Arrow thickness reflects magnitude of Ca^{2+} flux. Cav3, caveolin-3; β-AR, β-adrenergic receptor; RyR, ryanodine receptor; FKBP, FK506-binding protein; PLB, phospholamban; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase.

the eNOS isoform. In another rat model, both transient cerebral ischaemia and LPS could protect against later ischaemia.[112] LPS treatment was associated with increased eNOS expression, and protection was blocked by L-NAME. The anesthetics isoflurane and halothane also protect against cerebral ischaemia 24 h later.[113] iNOS induction is required and protection is blocked by the iNOS inhibitor aminoguanidine.

Cerebral IPC is more difficult to study in mice for technical reasons because the vessels are smaller. However, a mouse model of rapid preconditioning has been developed.[114] Three episodes of transient middle cerebral artery occlusion (the preconditioning stimulus), each lasting five minutes, protect the brain against damage from permanent vessel occlusion thirty minutes later. Infarct size measured 24 h later is reduced in preconditioned animals. This mouse model allows the study of NOS knockout mice to determine the separate roles of NOS isoforms. While wild-type mice demonstrate a reduction in infarct size following 3 cycles of IPC, neither eNOS knockout mice nor nNOS knockout mice do.[115] Baseline absolute blood flow measurements are the same in the three genotypes, so differences in the baseline absolute rCBF do not account for the results. Relative blood flow measurements by laser Doppler flowmetry confirm effective MCA occlusion with each preconditioning episode in each of the three genotypes. These results suggest that both nNOS and eNOS are required for cerebral IPC.

There are several potential mechanisms by which NO mediates IPC.[105] First, NO may be required as a trigger to stimulate downstream steps involved in the mechanisms of IPC. Second, NO may be involved as a mediator of protection by affecting neuronal resistance to ischaemic phenomena. NO interacts with at least two signaling pathways important to neuronal survival: the Ras/Raf/MEK/ERK cascade,[116,117] and the PI3 kinase/Akt pathway.[118,119] These pathways may be unifying mechanisms that underlie protection. Third, as a vasodilator, NO may augment blood flow by vasodilation, reducing leukocyte-endothelial interactions and platelet-endothelial interactions. Together these effects would limit the functional effect of ischaemia.

In the heart, NO is thought to play important protective roles, not only through blood flow effects, but also by directly enhancing cardioprotection. Myocardial ischaemia-reperfusion injury, and cardiac IPC appear to depend on iNOS, rather than nNOS or eNOS.[120,121] However, the molecular mechanisms of iNOS protection are not fully understood, and may involve electron transport or the mitochondrial permeability transition pore. iNOS knockout animals may be useful tools to define these processes.

14. Cardiac contractility

Pharmacologic blockade of NOS activity first suggested that NO plays a significant role in regulating cardiac contractility.[122–125] NO is produced in at least two major cell types in the heart: endothelial cells and cardiac myocytes. Endothelial cells are rich in eNOS and line the vasculature and endocardium. Cardiac myocytes, which are essential for cardiac excitation-contraction coupling, express both eNOS and nNOS. In cardiac myocytes, eNOS is localized to the sarcolemmal caveolae[126] through its interaction with caveolin-3.[127] nNOS, however, is localized to the sarcoplasmic reticulum,[128] where it is associated with the ryanodine receptor.[127] Because eNOS and nNOS reside in different subcellular locations, they can play distinct roles in cardiac function (see *Figure 2*).

Using Langendorff isolated heart preparations and *in vivo* measurements, Gyurko *et al*. found that eNOS knockout mice show no difference in basal contractility, but they do show enhanced inotropic and lusitropic responses to isoproterenol stimulation.[129] These findings were confirmed by further *in vivo* studies.[127] eNOS gene deletion enhances inotropic response not only to isoproterenol stimulation, but

also in basal conditions.[127] These results suggest that eNOS blunts the inotropic response to isoproterenol stimulation and its genetic absence enhances contractility.

nNOS knockout mice have been studied for phenotypes related to cardiac contractility, with variable results. In one case, nNOS knockout mice, as well as pharmacologic inhibition of nNOS in wild-type mice, causes enhanced basal LV contraction.[130,131] In another study, the same exon 2 nNOS knockout mice did not show significant differences in basal contractility, but showed decreased inotropic response to isoproterenol stimulation.[127] nNOS gene deletion has also been associated with more severe left ventricular remodelling after myocardial infarction.[132]

The subcellular localization of nNOS to the sarcoplasmic reticulum suggests that NO can modulate cardiomyocyte calcium handling. There is direct evidence that nNOS nitrosylates and activates the ryanodine receptor. Thus, nNOS normally maintains function of the ryanodine receptor, and in its absence, contractility will be affected. In patients with congestive heart failure, there is also evidence that nNOS is not properly localized to the sarcoplasmic reticulum.[133] When this occurs, nNOS may translocate to the L-type calcium channel at the cell surface, and play an inhibitory role much like eNOS does. The importance of nNOS to cardiac contractility and calcium handling is underscored by the recent finding that long QT syndrome, associated with dangerous ventricular arrhythmias in humans, is closely associated with NOS1-activating protein (NOS1AP), which binds to nNOS.[134] This raises the possibility that NOS1AP and nNOS affect not only cardiac contractility, but also propensity to ventricular arrhythmias through effects on the ryanodine receptor and calcium handling.

15. Non-cardiovascular phenotypes

In addition to the cardiovascular phenotypes discussed here, mutant mice have been useful to define the roles of each NOS isoform in other biological processes. nNOS knockout mice, eNOS knockout mice and nNOS/eNOS double knockout mice revealed complex roles for NO as a retrograde messenger in long term potentiation.[135,136] In addition to known roles of iNOS in osteoclast function, eNOS knockout mice show that eNOS is essential to bone formation and normal osteoblast activity.[137] Given the original enlarged gastrointestinal phenotype of nNOS knockout mice, it is not surprising that they have revealed how NO acts as an neurotransmitter to modulate the inhibitory junctional potential in the stomach and intestines.[138]

16. Conclusion

Targeted disruption of the nNOS, iNOS and eNOS genes in mice has led to the development of mutant mice that have been useful tools with which to study how NO affects blood pressure regulation, endothelial dysfunction, response to vascular injury, response to stroke and cerebral ischaemia, diet-induced atherosclerosis and cardiac contractility. As we begin to understand better the many diverse roles of NO, we can build upon this foundation to translate these findings into novel clinical approaches to prevent and treat cardiovascular diseases. Because patients with cardiovascular diseases are not totally devoid of either nNOS or eNOS, future work will likely involve more refined approaches to modulate the activity of NOS isoforms short of total gene disruption. Candidate regulatory sites and domains can be mutated, and the effects of these modifications can be studied *in vivo*. Perspectives from biochemistry, molecular biology, cell biology, physiology, chemistry and pharmacology will all complement genetic approaches to studying the roles of NO in cardiovascular diseases.

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