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INVITED REVIEW

Nitric oxide synthases in the pathogenesis of cardiovascular disease

Lessons from genetically modified mice

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Abstract Nitric oxide (NO) is produced in almost all tissues and organs, exerting a variety of biological actions under both physiological and pathological conditions. NO is synthesized by three distinct NO synthase (NOS) isoforms (neuronal, inducible, and endothelial NOS), all of which are expressed in the human cardiovascular system. Although the regulatory roles of NOSs in cardiovascular diseases have been described in pharmacological studies with selective and non-selective NOS inhibitors, the specificity of the NOS inhibitors continues to be an issue of debate. To overcome this issue, genetically engineered animals have been used. All types of NOS gene-deficient animals, including singly, doubly, and triply NOS-deficient mice, and various types of NOS gene-transgenic (TG) animals, including conditional and non-conditional TG mice bearing endothelium-specific or cardiomyocyte-specific overexpression of each NOS gene, have thus been developed. The roles of individual NOS isoforms as well as the entire NOS system in the cardiovascular system have been extensively investigated in those mice, providing pivotal insights into an understanding of the pathophysiology of NOSs in human cardiovascular diseases. Based on studies with the murine NOS genetic models, this review briefly summarizes the latest knowledge of NOSs and cardiovascular diseases.

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Introduction

Nitric oxide (NO) plays an important role in maintaining cardiovascular homeostasis through multiple biological actions [12, 21, 48, 65, 76, 78]. NO is formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citrulline. The NOS system consists of three distinct isoforms, including neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3).

It was initially demonstrated that nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small and physiological amount of NO in a calcium-dependent manner both under basal conditions and upon stimulation, whereas iNOS is induced only when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner [12, 21, 48, 65, 76, 78]. However, it was subsequently demonstrated that nNOS and eNOS are also subject to expressional regulation [9, 11, 51, 52, 73] whereas iNOS also could be constitutively expressed even under physiological conditions [4, 57].

Genetically engineered animals are a powerful experimental tool to study the function of target genes in vivo. All types of NOS gene-deficient animals, including singly, doubly, and triply NOS-deficient mice, have been developed (Table 1) [13, 14, 18, 19, 35, 38, 44, 56, 64, 68, 72, 83]. Furthermore, various types of NOS gene-transgenic (TG) animals, including conditional and non-conditional

Table 1 Mice lacking the NOS genes that have thus far been established

$NOS^{-/-}$ mice	Sites of gene deletion	References
$nNOS^{-/-}$	Exon 2 (#1)	[18]
	Exon 6	[14]
	Exon 6	[56]
$iNOS^{-/-}$	Proximal 585 bases of promoter plus exons 1–4 (#2)	[38]
	Near exons 1–5	[83]
	Exons 12 and 13 and a part of exon 11 (#3)	[35]
$eNOS^{-/-}$	Exons 24–26 (#4)	[19]
	Exon 12 (#5)	[64]
	Exons 24 and 25	[13]
$n/iNOSs^{-/-}$	#1 and #3	[72]
	#1 and #2	[44]
$n/eNOSs^{-/-}$	#1 and #4	[68]
	#1 and #5	[72]
	#1 and #4	[44]
$i/eNOSs^{-/-}$	#3 and #5	[72]
	#2 and #4	[44]
$n/i/eNOSs^{-/-}$	#1, #2 and #4	[44]

(Modified from Ref. [76] with a permission)

NOS nitric oxide synthase, *nNOS* neuronal NOS, *iNOS* inducible NOS, *eNOS* endothelial NOS

TG mice with endothelium-specific or cardiomyocyte-specific overexpression of each NOS isoform, have also been established (Table 2) [3, 5, 16, 22, 37, 46, 53, 55, 70, 77]. By using those genetically modified mice, the cardiovascular roles of NOSs have been extensively studied, and the findings provide important insights into the significance of NOSs in human cardiovascular diseases. In this review, we briefly summarize the current knowledge of NOSs and cardiovascular diseases on the findings obtained from the NOS gene-modified mice.

Vascular lesion formation

Role of eNOS

Endothelium-specific eNOS-TG mice with an 8-fold increase in vascular NOS activity showed decreased neointimal formation after carotid artery ligation [25] and another strain of endothelium-specific eNOS-TG mice with a 10-fold increase in vascular NOS activity similarly exhibited a reduction in atherosclerotic vascular lesion formation induced by breeding with $ApoE^{-/-}$ mice [77]. Consistent with these findings, $eNOS^{-/-}$ mice showed increased neointimal formation, accelerated medial thickening, and abnormal vascular remodeling in response to permanent carotid artery ligation (Fig. 1) [45, 85] and cuff placement around the femoral artery [59]. Furthermore, $eNOS^{-/-}/ApoE^{-/-}$ mice had accelerated formation of atherosclerotic vascular lesions as compared with $ApoE^{-/-}$ mice [26, 30]. These lines of evidence indicate vasculoprotective roles of eNOS in the pathogenesis of atherosclerotic vascular lesion formation. In contrast, in endothelium-specific eNOS-TG mice with an 8-fold increase in vascular NOS activity, atherosclerotic vascular lesion formation is accelerated when crossbred with $ApoE^{-/-}$ mice, where

Table 2 Mice overexpressing the NOS gene that have thus far been established

NOS-TG mice	Overexpression site	Promoter used	References
nNOS-TG	Myocardium(conditional)	α-MHC	[5]
	Myocardium(conditional)	α-MHC	[37]
	Brain	CaMKIIα	[55]
iNOS-TG	Myocardium(conditional)	α-MHC	[46]
	Myocardium	α-MHC	[16]
	Pancreatic β cell	Insulin	[70]
eNOS-TG	Endothelium	Preproendothelin-1	[53]
	Endothelium	eNOS	[77]
	Myocardium	α-MHC	[3]
	Myocardium	α-MHC	[22]

(Modified from Ref. [76] with a permission)

TG transgenic, *MHC* myosin heavy chain, *CaMKII* calcium-calmodulin multifunctional kinase II

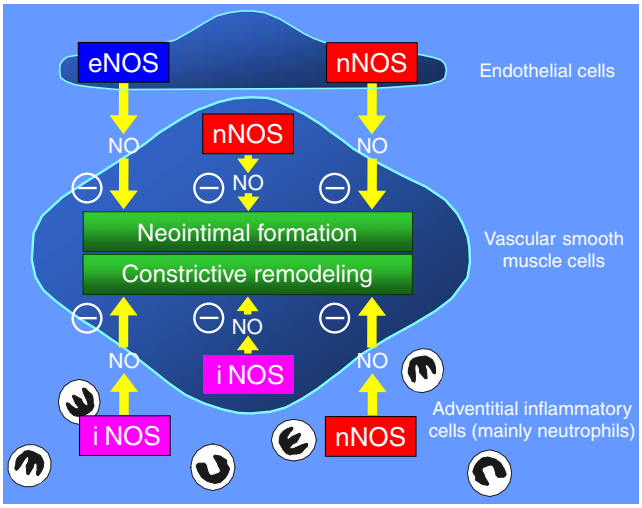

Fig. 1 The different vasculoprotective roles of three nitric oxide synthase (NOS) isoforms in a mouse carotid artery ligation model. Studies with each NOS isoform-deficient mice demonstrated that endothelial NOS (eNOS) inhibits neointimal formation, that inducible NOS (iNOS) attenuates constrictive vascular remodeling, and that neuronal NOS (nNOS) suppresses both neointimal formation and constrictive vascular remodeling. Thus, individual NOS isoforms have different vasculoprotective actions against vascular lesion formation in mice in vivo.–inhibition. (Modified from Ref. [76] with a permission)

enhanced oxidative stress due to relative tetrahydrobiopterin deficiency and development of a tolerance of the vascular smooth muscle to NO were demonstrated [54].

In eNOS$^{-/-}$ mice, in addition to the abolishment of NO-mediated relaxations, endothelium-dependent responses (relaxations and hyperpolarizations) mediated by endothelium-derived hyperpolarizing factor (EDHF) are markedly reduced [40]. Furthermore, the EDHF-mediated responses also are sensitive to catalase [40] and also are markedly reduced in Cu,Zn-SOD$^{-/-}$ mice [42]. These lines of evidence indicate that endothelium-derived hydrogen peroxide (H_2O_2), which is formed through dismutation of eNOS-derived superoxide anions by Cu,Zn-SOD, is an EDHF [40, 42, 66], although it is highly possible that several different factors and mechanisms other than endothelium-derived H_2O_2 derived from NOSs are also involved in the EDHF-mediated responses.

Role of iNOS

The role of iNOS in vascular lesion formation seems to be complicated. Deletion of the iNOS gene in mice exacerbated pathological vascular remodeling in a carotid artery ligation model (Fig. 1) [85] and in a cardiac transplant model [27]. However, the iNOS deletion conversely ameliorated neointimal formation in a carotid cuff placement model [7] and lipid-rich atherosclerotic vascular lesion formation in ApoE$^{-/-}$ mice [29]. Thus, iNOS appears to have two faces, which could be explained in part by the oxidant and antioxidant properties of iNOS. Indeed, NOSs produce superoxide anions rather than NO, with a resultant production of a potent oxidant peroxynitrite, under certain pathological conditions such as deficiency of a substrate (e.g., L-arginine) or a cofactor (e.g., tetrahydrobiopterin) termed as pathological NOS uncoupling [80, 81].

Role of nNOS

Although the regulatory roles of eNOS and iNOS in vascular lesion formation have been widely studied, little has been known about the role of nNOS. However, the expression of nNOS also is up-regulated in the neointima, endothelial cells, and macrophages in both early and advanced human atherosclerotic lesions [84]. We demonstrated that in nNOS$^{-/-}$ mice neointimal formation and constrictive vascular remodeling (a reduction in vascular cross-sectional area) following carotid artery ligation are accelerated (Fig. 1) [43]. In agreement with our finding, nNOS$^{-/-}$/ApoE$^{-/-}$ mice showed accelerated atherosclerotic vascular lesion formation as compared with ApoE$^{-/-}$ mice [31]. Up-regulation of nNOS may play a compensatory role in the presence of reduced eNOS activity (e.g., inflammation and arteriosclerosis) to maintain vascular homeostasis [73]. Furthermore, we demonstrated that inflammatory and proliferative stimuli and statins increase vascular nNOS expression [51, 52]. It also has been reported that hypoxic conditions [82] and hypertension [2] up-regulate vascular nNOS expression.

Role of the whole NOSs system

Because all NOSs play a role in the vascular system, we next conceived a project to investigate the roles of the whole NOS system in vivo. The roles of the NOS system in the human body have been investigated in pharmacological studies with non-selective NOS inhibitors and in studies with NOS isoform-deficient mice. However, because of both the non-specificity of the NOS inhibitors and compensation among the three NOS isoforms, the authentic roles of the NOS system were still poorly understood. To address this important issue, we have recently developed mice in which the entire NOS system is completely disrupted (triply nNOS/iNOS/eNOS-deficient mice) [44, 50]. The triply n/i/eNOS$^{-/-}$ mice, but not any singly NOS$^{-/-}$ mice, spontaneously develop arteriosclerotic vascular lesions (neointimal formation, medial thickening, and perivascular fibrosis) in the coronary and renal arteries, and lipid-rich atherosclerotic vascular lesions in the aorta, even on a normal chow diet [50, 74]. These results provided the first direct evidence for a vasculoprotective role of the entire NOS system in atherosclerosis.

Spontaneous myocardial infarction

Myocardial infarction (MI) is the leading cause of death for both genders worldwide [1, 28]. However, the molecular mechanisms for the pathogenesis of MI remain to be fully elucidated.

Role of the whole NOSs system

Although eNOS has potent vasculoprotective effects, [12, 21, 48, 65, 76, 78] neither deletion of the eNOS gene nor pharmacological inhibition of eNOS activity induces MI in animals. In contrast, our triply n/i/eNOSs$^{-/-}$ mice spontaneously develop MI and sudden cardiac death (Fig. 2a,b) [50, 74, 75]. This is the first in vivo demonstration of the protective roles of NOS synthases in the pathogenesis of MI. In our triply n/i/eNOSs$^{-/-}$ mice, arteriosclerosis is noted in most of the vasculature, whereas atherosclerosis is observed in the aorta alone. In humans, MI is caused not only by coronary atherosclerosis, but also by other mechanisms, including coronary intimal inflammation and coronary vasospasm [1, 79]. In the triply n/i/eNOSs$^{-/-}$ mice that died of MI, marked coronary intimal hyperplasia and medial thickening were noted (Fig. 2b,c). Furthermore, in the dead triply n/i/eNOSs$^{-/-}$ mice, marked infiltration of mast cells at the coronary artery adventitia was observed (Fig. 2d). Histamine released from adventitial mast cells is thought to cause coronary vasospasm with a resultant MI in humans [32]. It is thus possible that coronary arteriosclerosis and coronary vasospasm are involved in the cause of MI and death in the triply NOSs$^{-/-}$ mice (Fig. 3) [50, 74]. In our triply n/i/eNOSs$^{-/-}$ mice, not only NO-mediated but also EDHF-mediated endothelium-dependent relaxations are abolished [69] in addition the enhanced contractions to phenylephrine [74]. These vascular dysfunctions may also

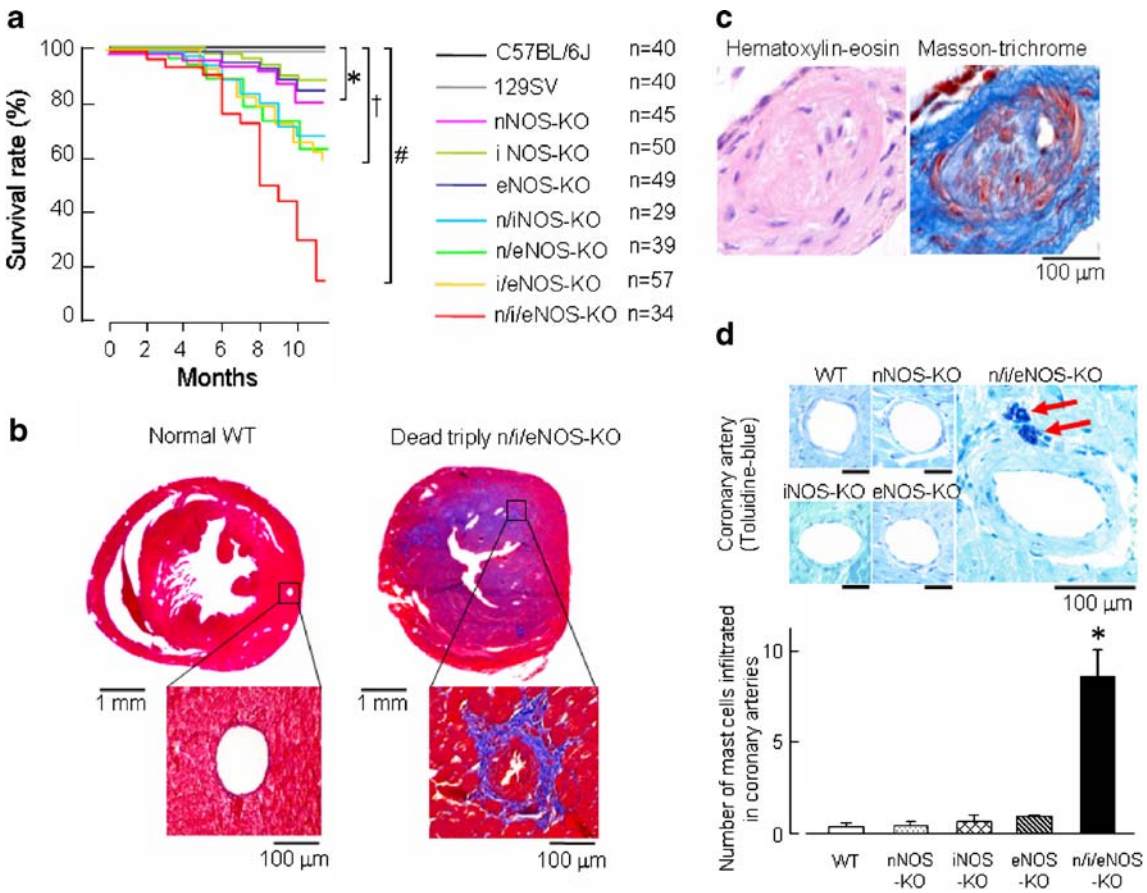

Fig. 2 Decreased survival, spontaneous myocardial infarction (MI), coronary arteriosclerosis and mast cell infiltration in male triply n/i/eNOSs$^{-/-}$ mice. **a** Survival rate (n=29–57). A *red line* represents markedly reduced survival in the triply n/i/eNOSs$^{-/-}$ mice. *, †, and #P<0.05 between wild-type (WT) C57BL/6J vs. singly, doubly, and triply NOS-KO, respectively. **b** Acute MI and coronary arteriosclerotic lesion formation in the triply n/i/eNOSs$^{-/-}$ mouse that died at 8 months of age (Masson-trichrome staining). *Blue* in the heart cross-section of the dead triply n/i/eNOSs$^{-/-}$ mouse indicates antero-septal acute MI. Adjacent coronary artery shows marked luminal narrowing, wall thickening, and perivascular fibrosis (*blue*). **c** Arteriosclerotic lesion formation in serial sections of the infarct-related coronary artery. **d** Mast cell infiltration in the coronary artery adventitia (toluidine-blue staining) n=10–33. *Red arrows* indicate mast cells. *P<0.05 vs. WT. (Reproduced from Ref. [76] with a permission)

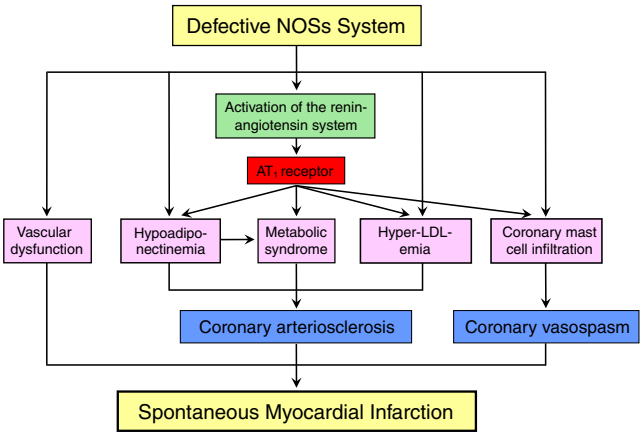

Fig. 3 Mechanisms for spontaneous MI caused by the defective NOS system in mice in vivo. Genetic disruption of all NOSs causes metabolic syndrome, hypoadiponectinemia, hyper-low-density-lipoprotein (LDL)-emia, coronary adventitial mast cell infiltration, and vascular dysfunction. These factors could contribute to the pathogenesis of spontaneous MI. Importantly, long-term pharmacological blockade of the AT_1 receptor significantly reduces the incidence of MI, along with amelioration of these risk factors. Thus, it is possible that the AT_1 receptor pathway is substantially involved in the molecular mechanisms of the pathological phenotypes and MI in the triply n/i/eNOSs$^{-/-}$ mice. (Modified from Ref. [76] with a permission)

be involved in the pathogenesis of MI in the triply NOSs$^{-/-}$ mice (Fig. 3).

Metabolic syndrome

Metabolic syndrome (MetS) is defined as a pathological state with accumulated cardiovascular risk factors of metabolic origin, including visceral obesity, hypertension, dyslipidemia, impaired glucose tolerance, and insulin resistance [49, 71]. Importantly, accumulation of three or more risk factors dramatically increases the risk of morbidity of atherosclerotic cardiovascular diseases by 11-fold, indicating that MetS is an important therapeutic target for the prevention and treatment of cardiovascular diseases [49, 71].

Roles of eNOS and the whole NOSs system

eNOS$^{-/-}$ and our triply n/i/eNOSs$^{-/-}$ mice manifest phenotypes that closely resemble MetS in humans [50, 74, 76]. Although the extent of each of cardiovascular risk factors (hypertension, dyslipidemia, and visceral obesity) was comparable between the 2 genotypes, the extent of impaired glucose tolerance and that of insulin resistance were greater in the triply n/i/eNOSs$^{-/-}$ than in the eNOS$^{-/-}$ mice, and hyper-low-density-lipoproteinemia was observed only in the triply n/i/eNOSs$^{-/-}$ mice [50]. Thus, it is possible that the whole NOSs system plays important roles in the prevention of MetS [50]. Although metabolic risk factors are present in the two genotypes, spontaneous MI is noted only in the triply n/i/eNOSs$^{-/-}$ mice. This discrepancy may be related to a compensatory mechanism by other NOSs that are not genetically disrupted [68]. Indeed, in the eNOS$^{-/-}$ mice, up-regulation of vascular nNOS expression has been reported [17, 33]. Furthermore, we also have demonstrated that NOS activity and plasma NOx levels are fairly well preserved in the singly eNOS$^{-/-}$ mice [44].

Adiponectin is an anti-atherogenic adipocytokine, which improves dyslipidemia, glucose metabolism, and insulin resistance, and inhibits the progression of atherosclerosis [24, 41, 67]. Under the condition of obesity with adipocyte hypertrophy, the synthesis of adiponectin is decreased and in patients with MetS, the circulating levels of adiponectin are also reduced, in contrast to the increases in other atherogenic adipocytokine levels. This adiponectin deficiency is thought to play a pivotal role in the pathogenesis of MetS and its vascular complications [41]. In our triply n/i/eNOSs$^{-/-}$ mice, plasma adiponectin levels were significantly reduced [50]. Thus, the adiponectin deficiency may contribute to the development of metabolic abnormalities and arteriosclerotic lesion formation in the triply n/i/eNOS$^{-/-}$ mice (Fig. 3).

Importantly, the renin-angiotensin system is markedly activated in the triply n/i/eNOSs$^{-/-}$ mice, and long-term treatment with an angiotensin II type 1 (AT1) receptor blocker, olmesartan, potently inhibit coronary arteriosclerotic lesion formation, adventitial mast cell infiltration, and the occurrence of MI in the mice, with a resultant improvement in prognosis [50]. Furthermore, the long-term treatment with olmesartan reverses all the abnormal metabolic phenotypes, together with amelioration of hypoadiponectinemia [50]. These results suggest that the AT1 receptor pathway is substantially involved in the pathogenesis of MI in our triply n/i/eNOSs$^{-/-}$ mice (Fig. 3).

Heart failure

Role of eNOS

In cardiomyocyte-specific eNOS-TG mice with a 30-fold increase in cardiac NOS activity, left ventricular (LV) remodeling after coronary artery ligation is suppressed, showing improved LV systolic and diastolic function and attenuation of LV hypertrophy [22]. Endothelium-specific eNOS-TG mice with a 12-fold increase in vascular NOS activity also exhibit improved survival, LV dysfunction, and pulmonary edema following coronary ligation without affecting LV remodeling [23]. Consistent with these findings, eNOS$^{-/-}$ mice with heart failure (HF) due to

either MI [63] or pressure overload [20] show reduced survival and exacerbation of LV remodeling and LV dysfunction. It also has been reported that eNOS mediates, at least in part, the beneficial cardiovascular protective effects of statins [34], angiotensin converting enzyme inhibitors [36], AT1 receptor blockers [36], and corticosteroids [15] in experimental HF. Thus, it is evident that eNOS plays important protective roles in HF [39, 58].

Role of nNOS

Conditionally targeted cardiomyocyte-specific nNOS-TG mice with a 5-fold increase in cardiac NOS activity showed delayed transition toward HF in response to pressure overload [37]. In agreement with this evidence, two strains of $nNOS^{-/-}$ mice with MI-induced HF similarly showed reduced survival and exacerbation of pathological LV remodeling or dysfunction after coronary artery ligation, although not totally identical in the two strains [8, 62]. Thus, it is possible that in addition to eNOS, nNOS also plays a cardioprotective role in HF [6].

Role of iNOS

Increased iNOS expression is noted in cardiomyocytes in septic shock, myocarditis, ischemia, and dilated cardiomyopathy, and has been implicated in the development of HF. However, cardiomyocyte-specific iNOS overexpression per se (in two different strains with either a 10-fold [46] or 40-fold increase [16] in cardiac NOS activity) did not cause HF, suggesting that increased iNOS expression per se is not the triggering mechanism in HF. In contrast, $iNOS^{-/-}$ mice with HF induced by MI [10, 60] and by pressure overload [86] showed improved survival, less LV remodeling and dysfunction, and decreased myocardial apoptosis. Furthermore, $iNOS^{-/-}$ mice with HF induced by cardio-specific overexpression of TNF-α show improved β-adrenergic inotropic responsiveness. Thus, it is possible that, in contrast to eNOS and nNOS, iNOS exerts opposite and unfavorable effects in HF. The underlying mechanisms for the contrasting roles of NOS isoforms in HF are unclear, but may relate to the differences in their spatial localization, expressional regulation, NO-generating capacity, and peroxynitrite generation [47, 58, 61].

Other forms of cardiovascular diseases

A lines of accumulating evidence also has suggested that the impairment of the NOSs system is involved in the pathogenesis of other forms of cardiovascular diseases, including aortic aneurysms, arrhythmias, and congenital heart disease [76].

Conclusions

The mouse is the most ideal genetically modifiable mammalian presently available. Studies with mice that are deficient of or overexpressing NOSs provide pivotal insights into the roles of NOSs in the pathogenesis of cardiovascular diseases. In general, eNOS and nNOS exert cardiovascular protective roles, while iNOS seems to exert dual effects in the cardiovascular system. The observations with the genetically modified animals have greatly advanced our understanding of the roles of the NOSs system in the pathogenesis of human cardiovascular diseases. Further studies are certainly needed to clarify whether these observations can be translated to human patients with cardiovascular diseases.

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