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Critical review

Significance of nitric oxide synthases: Lessons from triple nitric oxide synthases null mice

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ABSTRACT

Nitric oxide (NO) is synthesized by three distinct NO synthases (neuronal, inducible, and endothelial NOSs), all of which are expressed in almost all tissues and organs in humans. The regulatory roles of NOSs in vivo have been investigated in pharmacological studies with non-selective NOS inhibitors. However, the specificity of the inhibitors continues to be an issue of debate, and the authentic significance of NOSs is still poorly understood. To address this issue, we generated mice in which all three NOS genes are completely disrupted. The triple NOSs null mice exhibited cardiovascular abnormalities, including hypertension, arteriosclerosis, myocardial infarction, cardiac hypertrophy, diastolic heart failure, and reduced EDHF responses, with a shorter survival. The triple NOSs null mice also displayed metabolic abnormalities, including metabolic syndrome and high-fat diet-induced severe dyslipidemia. Furthermore, the triple NOSs null mice showed renal abnormalities (nephrogenic diabetes insipidus and pathological renal remodeling), lung abnormalities (accelerated pulmonary fibrosis), and bone abnormalities (increased bone mineral density and bone turnover). These results provide evidence that NOSs play pivotal roles in the pathogenesis of a wide variety of disorders. This review summarizes the latest knowledge on the significance of NOSs in vivo, based on lessons learned from experiments with our triple mutant model.

1. Introduction

Nitric oxide (NO) plays a crucial role in maintaining homeostasis (1–4). NO is synthesized from its precursor L-arginine by a family of NO synthases (NOSs) that include neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS). It was initially reported that nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a calcium-dependent manner under basal conditions and upon stimulation, and that iNOS is induced only when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner (3,4). However, recent studies have revealed that nNOS and eNOS are also subject to expressional regulation (5–9), and that iNOS is expressed even under physiological conditions (10,11). Thus, it has become evident that all three NOS isoforms are expressed under both physiological and pathological conditions (10,12).

The roles of NO derived from whole NOSs have been examined in pharmacological studies with non-selective NOSs inhibitors, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA). However, the NOS inhibitors possess multiple non-specific actions, including antagonism of muscarinic acetylcholine receptors (13), generation of superoxide anions (14),

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inhibition of cytochrome c reduction (15), and inhibition of endothelium-independent relaxation induced by amiloride or cAMP (16). We also reported that vascular lesion formation caused by long-term treatment with L-NAME or L-NMMA is not mediated by the simple inhibition of eNOS in mice, and that activation of the tissue renin-angiotensin system and increased oxidative stress are involved in the long-term vascular effects of the L-arginine analogues in an NO-independent manner (17,18).

The roles of NO derived from whole NOSs have also been investigated in studies with mice that lack each NOS isoform. However, although the single eNOS null mice manifest accumulation of cardiovascular risk factors that mimic human metabolic syndrome (19), and although it is well established that eNOS exerts anti-arteriosclerotic effects (20–25), the single eNOS null mice do not spontaneously develop arteriosclerotic/atherosclerotic vascular lesion formation (26). This inconsistency could be due to a compensatory mechanism by other NOSs that are not genetically disrupted (27). Indeed, in the singly eNOS$^{-/-}$ mice, up-regulation of vascular nNOS expression has been indicated (28,29). Furthermore, we revealed that NOS activity and NOx (nitrite plus nitrate) production are fairly well preserved in that genotype (30). Thus, the authentic roles of endogenous NO derived from entire NOSs still remain to be fully elucidated.

To address this important issue, we successfully developed mice in which all three NOS genes are completely disrupted (30). The expression and activity of NOSs are totally absent in the triple n/i/eNOSs null mice before and after administration of lipopolysaccharide. While the triple NOSs null mice were viable and appeared normal, their survival and fertility rates were markedly reduced as compared with wild-type mice. The triple NOSs null mice exhibited phenotypes in the cardiovascular, metabolic, renal, respiratory, and bone systems. These results provide evidence that NOSs play pivotal roles in the pathogenesis of a wide variety of disorders. This review summarizes the latest knowledge on the significance of NOSs in vivo, based on lessons we learned from experiments with our triple mutant model.

2. Significance of NOSs in the cardiovascular system

2.1. Hypertension

The triple NOSs null mice were significantly hypertensive as compared with the wild-type mice (30). The degree of hypertension in the triple NOSs null mice was similar to that in the eNOS null and eNOS gene-disrupted double NOSs null mice (Fig. 1A). These results suggest that hypertension is a common characteristic of the eNOS gene disruption and is caused by a lack of endothelium-derived NO with a resultant increase in peripheral vascular resistance (31). Heart rate was significantly lower in the triple NOSs null than in the wild-type mice, and the degree of bradycardia in the triple NOSs null mice was also equivalent to that in the eNOS gene-disrupted single and double NOSs null mice (Fig. 1B), indicating that bradycardia is also a common phenotype of the eNOS gene deletion. Although there is no conclusive explanation for the decreased heart rate in association with the eNOS gene deletion, previous studies revealed that eNOS-derived NO could affect baroreflex resetting or could be involved in establishing the baroreceptor setpoint (31).

2.2. Arteriosclerosis

We previously revealed that not only eNOS and iNOS but also nNOS is expressed in vascular lesions in a mouse carotid artery ligation model and a rat balloon injury model, and that all three NOSs play a role in the regulation of vascular lesion formation (7–9,32). Spontaneous development of vascular lesion formation (neointimal formation, medial thickening, and perivascular fibrosis) was noted in the large epicardial coronary arteries, coronary microvessels, and renal arteries in the triple NOSs null mice, but not in the eNOS null mice (2,33). Spontaneous lipid accumulation was also observed in the aorta of the triple NOSs null mice (2,33). These results suggest the crucial role of NOSs in inhibiting vascular lesion formation. The extent of hypertension was comparable in the triple NOSs null and eNOS null mice, whereas spontaneous vascular lesion formation was observed only in the triple

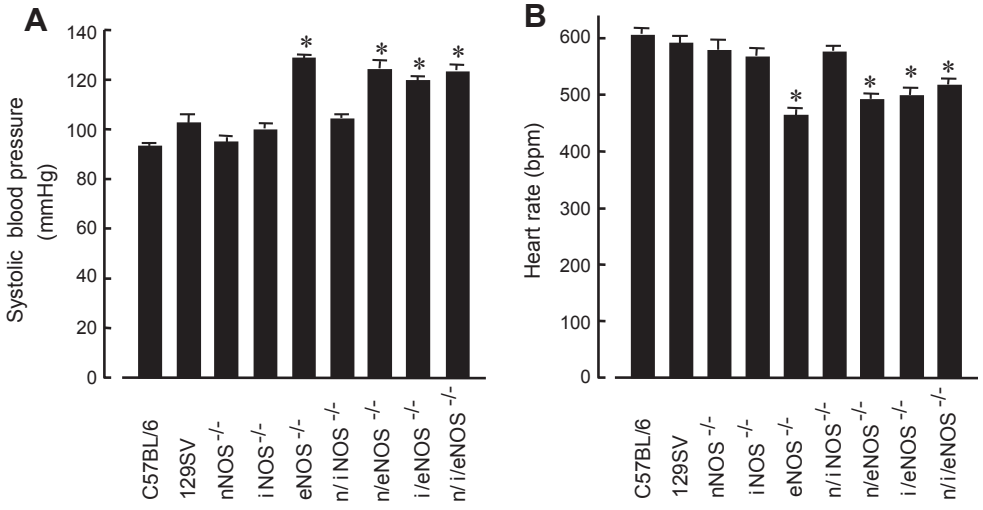

Fig. 1. Hemodynamics in wild-type and NOSs null mice. (**A**) Systolic blood pressure measured by the tail-cuff method under conscious conditions ($n = 9–16$). *$P < 0.05$ vs. wild-type C57BL/6 mice. (**B**) Heart rate measured by the tail-cuff method under conscious conditions ($n = 9–16$). *$P < 0.05$ vs. wild-type C57BL/6 mice. All single, double, and triple NOSs$^{-/-}$ mice are derived from both wild-type C57BL/6J and 129SV mice. nNOS$^{-/-}$ and eNOS$^{-/-}$ mice were backcrossed with C57BL/6J mice over five generations, while iNOS$^{-/-}$ mice were not backcrossed with any strains. Thus, we used both C57BL/6J and 129SV mice as wild genotype controls. We confirmed that there was no significant difference in blood pressure levels, heart rate, plasma lipid profile, glucose metabolism, or the amount of visceral adipose tissue between the C57BL/6 and 129SV mice at 3 months of age. Furthermore, we verified that there also was no significant difference in the extent of coronary vascular lesion formation between the 2 wild-type genotypes at 5 months of age. Results are expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 30 with permission.