Nitric oxide (NO) plays important roles in maintaining cardiovascular homeostasis.\textsuperscript{1–5} NO is formed from its precursor, \(\text{L}-\)arginine, by a family of NO synthases (NOSs) with stoichiometric production of \(\text{L}-\)citrulline. Three distinct NOS isoforms exist that are encoded by 3 distinct genes, including neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). Initial NO studies indicated 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 both under basal conditions and upon stimulation, and that iNOS is induced only when stimulated by microbial endotoxins or certain pro-inflammatory cytokines, producing a greater amount of NO in a calcium-independent manner.\textsuperscript{1–5} However, recent studies have revealed that both nNOS and eNOS are subject to expressional regulation, and that iNOS is constitutively expressed even under physiological conditions.\textsuperscript{4} In addition, it has become apparent that in addition to eNOS and iNOS, nNOS is also expressed in the cardiovascular system, exerting important cardiovascular actions.\textsuperscript{4}
The roles of the NOS system in vivo have been investigated in pharmacological studies. As pharmacological tools are used to inhibit NO synthesis, l-arginine analogues have been widely used. However, the l-arginine analogues possess multiple non-specific actions. Indeed, we also clarified the NO-independent vascular actions of l-arginine analogues (eg, a synthetic analogue, N^ω-nitro-l-arginine methyl ester, and an endogenous analogue, asymmetric dimethylarginine). Although long-term treatment with l-arginine analogues had long been believed without doubt to simply inhibit vascular NO synthesis and cause arteriosclerotic vascular lesion formation, we found that the long-term vascular effects of l-arginine analogues are not solely mediated by the simple inhibition of NO synthesis. Activation of the tissue renin-angiotensin system and increased oxidative stress, independent of endogenous NO inhibition, are involved in the long-term vascular effects of those analogues. These findings questioned the previous theory regarding the effects of l-arginine analogues and warranted re-evaluation of previous studies using those analogues. Thus, due to their non-specificity, the authentic roles of the NOS system in our body still remain to be fully elucidated.

To address this important issue, we have generated mice in which all 3 NOS isoforms are completely disrupted (triply n/i/eNOS−/− mice). The n/i/eNOS−/− mice are unexpectedly viable and appear normal, but their survival and fertility rates are markedly reduced as compared with wild-type (WT) mice. The n/i/eNOS−/− mice also exhibit nephrogenic diabetes insipidus. Furthermore, we have recently revealed that the n/i/eNOS−/− mice spontaneously develop cardiovascular diseases, including hypertension, dyslipidemia, and arteriosclerosis/atherosclerosis. Nevertheless, it remains to be determined whether or not the NOS system plays a role in the maintenance of the cardiac architecture and function. Thus, in this study, we tested our hypothesis that genetic disruption of the entire NOS system causes left ventricular (LV) hypertrophy (LVH) and cardiac dysfunction.

**Methods**

**Animal Preparation**

This study was reviewed and approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan, and was carried out according to the Institutional Guidelines for Animal Experimentation and the Law (No.105) and Notification (No.6) of the Japanese Government. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Two- and 5-month-old mice of both genders maintained on a regular diet (0.26 g sodium per 100 g diet) were used. Most experiments were performed in male mice, and female mice were used in experiments regarding gender difference. We generated the n/i/eNOS−/− mice by crossing doubly n/i/eNOS−/− mice, as previously reported. The n/i/eNOS−/− mice spontaneously develop cardiovascular diseases, including hypertension, dyslipidemia, and arteriosclerosis/atherosclerosis. Nevertheless, it remains to be determined whether or not the NOS system plays a role in the maintenance of the cardiac architecture and function. Thus, in this study, we tested our hypothesis that genetic disruption of the entire NOS system causes left ventricular (LV) hypertrophy (LVH) and cardiac dysfunction.

**Brain Natriuretic Peptide (BNP)**

The LV was homogenized with an isoen reagent (Nippon Gene, Tokyo, Japan) and total RNA was extracted. The RNA was converted to cDNA using a QuantiTect Reverse Transcription kit (Qiagen, Tokyo, Japan). Quantitative real-time PCR was performed with a QuantiTect Probe PCR kit (Quigen). Fluorescence resonance energy transfer probes and primers were purchased from Applied Biosystems (Foster City, CA, USA).

**Eight-Isoprostane**

Urine was collected with the use of metabolic cages and stored at –80°C. Urinary 8-isoprostane excretion was measured with an enzyme immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA).

**Morphology**

The animals were euthanized by an overdose of diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). The aorta was cannulated and perfused with a 4% paraformaldehyde solution under physiological pressure. The LV was embedded in paraffin, and 5 µm-thick slices were stained with hematoxylin-eosin or Masson-Trichrome solutions. The sections were scanned with a light microscope equipped with a 2-dimensional analysis system (IBAS, Carl Zeiss, Jena, Germany). The extent of myocardial fibrosis was evaluated by the ratio of collagen deposition area to total myocardial area.
Diastolic Dysfunction in Mice Lacking All NOSs

Tumor Necrosis Factor (TNF), Interferon (INF)-γ and Transforming Growth Factor (TGF)-β
Total RNA was isolated from the LV myocardium, and cardiac mRNA levels for TNF, INF-γ and TGF-β were analyzed by RNase protection assay (BD Biosciences PharMingen, Tokyo, Japan). The value of each hybridized probe was normalized to that of GAPDH in each template set as an internal control.

Statistical Analysis
Results are expressed as mean±SEM. Statistical analyses were performed by using a 1-way ANOVA followed by Fisher’s post-hoc test. A value of P<0.05 was considered to be statistically significant.

Results
LVH
To evaluate alterations in the cardiac structure and function in the NOS−/− mice, we performed pathological, echocardiographic, and hemodynamic analyses. At 2 months of age, no significant cardiac morphological or functional changes were detected in any strains studied (data not shown). However, at 5 months of age, significant concentric LVH (Figures 1A, 2A–C), increased LV weight/body weight ratio (Figure 1B), and cardiac myocyte hypertrophy (Figures 1C, D) were noted in the n/i/eNOS−/− and eNOS−/− mice, but not in the nNOS−/− or iNOS−/− mice, as compared with the WT mice. Importantly, the extents of those structural changes were both significantly larger in the n/i/eNOS−/− than in the eNOS−/− mice. The LV end-diastolic dimension was significantly smaller only in the n/i/eNOS−/− mice compared with the WT mice, indicating centripetal LVH in the n/i/eNOS−/− mice (Figure 2D). As cardiac abnormalities were seen in the 5-month-old mice, subsequent experiments were carried out on the mice of that age.

Blood Pressure
Arterial blood pressure (mmHg) was significantly higher in the n/i/eNOS−/− and the eNOS−/− mice, but not in the nNOS−/− or the iNOS−/− mice, as compared with the WT mice, and the hypertensive levels were comparable in the n/i/eNOS−/− and the eNOS−/− mice (Figure 2H).

LV Function
Fractional shortening (Figure 2E) and peak positive dP/dt
Figure 2. Centripetal left ventricular hypertrophy (LVH), diastolic dysfunction, and hypertension in n/I/eNOS−/− and eNOS−/− mice. (A) Representative trace of M-mode echocardiography. (B–E) M-mode echocardiographic parameters (n=5–6). (F) Representative trace of Doppler echocardiography. (G) E/A wave ratio (n=5–6). (H) Arterial blood pressure (n=12). NOS, nitric oxide synthase. *P<0.05 vs wild-type (WT), †P<0.05 vs eNOS−/−.
Diastolic Dysfunction in Mice Lacking All NOSs

(\(+\text{dP/dt}\), Figure 3A,B), which are echocardiographic and hemodynamic markers of LV systolic function, respectively, were unchanged in all the genotypes studied. However, the E/A wave ratio (Figure 2G), which is an echocardiographic marker of LV diastolic function, was significantly altered in the n/i/eNOS\(^{-/-}\) mice and to a lesser extent in the eNOS\(^{-/-}\) mice as compared with the WT mice. Furthermore, peak negative dP/dt (\(-\text{dP/dt}\), Figure 3C) and Tau (Figure 3D), both of which are hemodynamic markers of LV diastolic function, were also significantly different only in the n/i/eNOS\(^{-/-}\) mice compared with the WT mice. In addition, a significant increase in LV end-diastolic pressure (LVEDP) was noted only in the n/i/eNOS\(^{-/-}\) mice (Figure 3E). In contrast, no local wall motion abnormality was seen in any of the genotypes.

The above experiments were performed in male mice. Although female n/i/eNOS\(^{-/-}\) mice also showed similar cardiac phenotypes, there were no significant gender differences in the extent of LVH or diastolic dysfunction (data not shown).
Lung Weight
The lung wet weight/body weight ratio was significantly increased in the triply n/i/eNOS–/– mice as compared with the WT mice (Figure 4A).

BNP Level
Cardiac BNP mRNA expression was significantly higher in the n/i/eNOS–/– than in the WT mice (Figure 4B).

Myocardial Fibrosis
Significant myocardial fibrosis was observed in the n/i/eNOS–/– mice (Figures 4C, D).

TGF-β Level
Cardiac TGF-β mRNA expression was significantly higher in the n/i/eNOS–/– mice than in the WT mice (Figure 4E).

TNF, INF-γ, 8-Isoprostane and Malondialdehyde
Cardiac mRNA expression of TNF and INF-γ, proinflam-
Diastolic Dysfunction in Mice Lacking All NOSs

Inflammatory cytokines (Figures 5A, B), and urinary 8-isoprostane excretion and cardiac malondialdehyde levels, markers of oxidative stress (16) (Figures 5C, D), were significantly enhanced in the n/i/eNOS−/− mice as compared with the WT mice.

Effects of an AT1 Receptor Blocker

Finally, we tested our hypothesis that the AT1 receptor pathway is involved in the development of cardiac abnormalities in the n/i/eNOS−/− mice. Long-term (3 months) oral treatment with olmesartan, a selective and potent AT1 receptor blocker, significantly ameliorated LVH (Figures 6A; 7A–C), LV weight/body weight ratio (Figure 6B), cardiac myocyte hypertrophy (Figures 6C, D) and echocardiographic (E/A wave ratio) (Figures 7D, E) and hemodynamic parameters (−dp/dt, Tau, and LVEDP) (Figures 7G–I) in the n/i/eNOS−/− mice. Furthermore, long-term treatment with olmesartan also significantly reduced the lung wet weight/dry weight ratio (Figure 8A), BNP (Figure 8B), myocardial fibrosis (Figures 8C–E), proinflammatory cytokines (TNF and INF-γ) (Figures 8F, G), and oxidative stress marker levels (8-isoprostane and malondialdehyde) (Figures 8H, I) in the n/i/eNOS−/− mice.

Long-term (3 months) oral treatment with hydralazine, an antihypertensive drug, significantly reduced arterial blood pressure in the n/i/eNOS−/− mice to the same extent as with olmesartan (Figure 8J). However, the treatment with hydralazine did not significantly improve the morphological (Figure 6), echocardiographic (Figures 7A–E), or hemodynamic abnormalities (Figures 7F–I) of the n/i/eNOS−/− mice. In addition, the treatment with hydralazine did not significantly affect the lung wet weight/dry weight ratio (Figure 8A), BNP (Figure 8B), myocardial fibrosis (Figures 8C–E), proinflammatory cytokine (INF-γ) (Figure 8G), or oxidative stress marker levels (Figures 8H, I) in the genotype, although the effect on the cardiac TNF levels (Figure 8F) was an exception.
Discussion

The major novel findings of the present study were that the triply n/i/eNOS−/− mice spontaneously developed LVH and diastolic dysfunction, and that long-term treatment with olmesartan prevented those structural and functional alterations. These results demonstrate that the genetic disruption of all NOSs results in pathological LV remodeling and abnormal lusitropy by an AT1 receptor-dependent mechanism. This is the first study that demonstrates that the defective NOS system is linked to the pathogenesis of diastolic dysfunction.

Murine Model of Spontaneous Diastolic Heart Failure (HF)

HF is a leading cause of morbidity and mortality in industrialized countries.18,19 There is growing recognition that not only systolic HF but also diastolic HF with normal systolic function is common and causes significant morbidity and mortality. Indeed, recent studies have revealed that as many as 30–50% of patients with congestive HF have diastolic HF, and that the morbidity and mortality rates for diastolic HF are nearly identical to those for systolic HF in aged patients.20 Based on these new lines of evidence, diastolic HF has currently attracted considerable attention.

In this study, we evaluated diastolic HF of the n/i/eNOS−/− mice by pulse Doppler echocardiography and intra-LV pressure analysis. Tissue Doppler echocardiography and intra-LV pressure-loop measurement might be more reliable analytical procedures. However, we were able to indicate various abnormalities, including echocardiographic E/A wave ratio, hemodynamic –dP/dt and Tau, and LVEDP. Thus, it is likely that the n/i/eNOS−/− mice have diastolic HF.

Thus far, 4 genetically engineered mouse models that spontaneously develop diastolic dysfunction in the absence of systolic dysfunction have been reported: (1) mice lacking the...
Figure 7. Beneficial effects of long-term oral treatment with olmesartan (Olme), but not with hydralazine (Hyd), on (A–E) echocardiographic and (F–I) hemodynamic parameters of n/i/eNOS−/− mice (n=5–6). LVEDP, left ventricular end-diastolic pressure; LVP, left ventricular pressure; NOS, nitric oxide synthase. *P<0.05 vs control.
Figure 8. Beneficial effects of long-term oral treatment with olmesartan (Olme), but not with hydralazine (Hyd), on (A) lung wet weight/dry weight ratio (n=5–8), (C, D) cardiac fibrosis (n=5–8), (B, E–I) biochemical parameters (n=5–8), and (J) blood pressure (n=8–12) in n/i/eNOS−/− mice. Scale bars in (B) indicate 0.5 mm. INF, interferon; NOS, nitric oxide synthase; TNF, tumor necrosis factor. *P<0.05 vs control.
α1 subunit of soluble guanylate cyclase;21 (2) mice deficient in the peptide hormone, relaxin;20,22 (3) mice overexpressing cardiac ACE;23 and (4) mice bearing a R58Q mutation of the ventricular myosin regulatory light chain.24 However, no evidence of HF has been present in the former 2 mice groups, and indexes of HF (eg, LVEDP) have not been studied in the latter 2 mice groups. In contrast, we demonstrated that the n/i/eNOS−/− mice showed higher LVEDP and increased the lung wet weight in addition to diastolic dysfunction. Thus, our triply mutant mice might be the first genetically engineered murine model of spontaneous diastolic HF. In human patients with diastolic HF, the expression level of 3 NOS isoforms or the level of NO production has not been reported. Thus, the significance of the n/i/eNOS−/− mice as a model of human diastolic HF remains to be clarified in future studies.

NO attenuates cardiac myocyte hypertrophy and cardiac fibrosis in response to norepinephrine stimulation in cultured rat LV cells,25 and NO augments LV diastolic distensibility and myocardial relaxation in isolated mammalian beating hearts and in humans.26 Furthermore, an increase in cardiac eNOS expression induced by pharmacological treatment with the eNOS enhancer, AVE3085, has been shown to ameliorate diastolic HF in Dahl salt-sensitive rats. These results are in agreement with our evidence that loss of NO leads to cardiac myocyte hypertrophy, cardiac fibrosis, and diastolic function. Diastolic HF is prevalent in women and elderly adults, and is frequently associated with hypertension. Consistent with those human findings, in this study, diastolic dysfunction was seen in hypertensive older mice, although there was no gender difference. We previously reported that the n/i/eNOS−/− mice died due to myocardial infarction, renal diseases, and ileus, and that the cause of death in 10% of dead n/i/eNOS−/− mice was unknown.35 As pulmonary congestion was evident in all 10% of the mice, it is speculated that those mice might have died because of diastolic HF, although we can’t deny the possibility of postmortem change.

No Effect of Blood Pressure on Cardiac Phenotypes of n/i/eNOS−/− Mice

Whereas the extent of the increase in blood pressure was comparable in the n/i/eNOS−/− and eNOS−/− mice, the extent of LVH was greater in the n/i/eNOS−/− than in the eNOS−/− mice. Moreover, despite similar blood pressure levels, diastolic dysfunction was noted only in the n/i/eNOS−/− mice, but not in the eNOS−/− mice. In addition, antihypertensive treatment with hydralazine failed to inhibit the progression of LVH and diastolic dysfunction in the n/i/eNOS−/− mice. Thus, it is possible that hypertension plays a minor role in the occurrence of those phenotypes in the n/i/eNOS−/− mice.

Involvement of BNP and TGF-β in Cardiac Phenotypes of n/i/eNOS−/− Mice

It has been reported that plasma BNP concentrations can predict diastolic abnormalities in patients with normal systolic function.37 Consistent with the evidence, cardiac BNP levels were elevated in the n/i/eNOS−/− mice. Cardiac TGF-β levels were also upregulated in the n/i/eNOS−/− mice. TGF-β has been shown to increase the production of collagen type I and III, extracellular matrix proteins, and integrins, leading to cardiac myocyte fibrosis and growth.38 In addition, TGF-β has been indicated to induce the deposition of extracellular matrix proteins and to impair LV diastolic stiffness, contributing to subsequent diastolic HF.38 Thus, it is conceivable that TGF-β is involved in the development of cardiac fibrosis, LVH, and diastolic dysfunction in the n/i/eNOS−/− mice.

Involvement of Inflammation and Oxidative Stress in Cardiac Phenotypes of n/i/eNOS−/− Mice

Proinflammatory cytokines and reactive oxygen species are enhanced in LVH and/or HF.39,40 Those factors induce cardiac myocyte hypertrophy, apoptosis, and interstitial fibrosis, contributing to the progression of maladaptive cardiac remodeling and failure.41,42 In the present study, the levels of proinflammatory cytokines (ie, TNF and INF-γ) and oxidative stress markers (ie, 8-isoprostane and malondialdehyde) were increased in the n/i/eNOS−/− mice. In addition, our previous study reported higher superoxide anion generation in the heart of the n/i/eNOS−/− mice.39 It is thus suggested that inflammation and oxidative stress are also involved in the development of cardiac abnormalities in the n/i/eNOS−/− mice.

Role of AT1 Receptor Pathway in the Development of Cardiac Phenotypes in n/i/eNOS−/− Mice

We finally examined the molecular mechanism of the development of cardiac phenotypes in n/i/eNOS−/− mice. We previously revealed that the renin-angiotensin system, as evaluated by the tissue levels of angiotensin-converting enzyme and AT1 receptor and the plasma levels of renin and angiotensin II, was activated in the n/i/eNOS−/− mice.43 The renin-angiotensin system plays an important role in the pathogenesis of heart diseases.44,45 Based on the evidence, we further tested our hypothesis that the AT1 receptor signal transduction pathway mediates cardiac abnormalities in the n/i/eNOS−/− mice. Notably, long-term oral treatment with the AT1 receptor blocker, olmesartan, but not with the antihypertensive drug, hydralazine, potently improved LVH, cardiac myocyte hypertrophy, and diastolic dysfunction in the n/i/eNOS−/− mice, and was accompanied by reductions in LVEDP and lung wet weight. Furthermore, the long-term oral treatment with olmesartan also ameliorated the levels of BNP, cardiac fibrosis, TGF-β, inflammation, and oxidative stress in the n/i/eNOS−/− mice. The plasma concentration of olmesartan achieved by the olmesartan treatment that we used in this study has been shown to inhibit the binding of the AT1 receptor almost completely without affecting the AT2 receptor, indicating that olmesartan antagonizes the AT1 receptor both selectively and potently under our experimental conditions. It is thus evident that the AT1 receptor pathway plays a central role in the pathogenesis of cardiac disorders in the n/i/eNOS−/− mice.

Conclusion

In conclusion, we were able to prove that the complete deletion of all NOS genes causes spontaneous development of LVH and diastolic dysfunction associated with elevated LVEDP and enhanced lung wet weight in mice in vivo via the AT1 receptor pathway. The present findings should contribute to a better understanding of the role of the defective NOS system in the pathogenesis of diastolic HF. In the clinical setting, pharmacological treatment of diastolic HF has not yet been established. Our findings might offer important insights into the usefulness of the renin-angiotensin system inhibitors to treat human diastolic HF in the presence of reduced NO production.
Acknowledgments

This work was supported, in part, by Grants-in-Aid for Scientific Research (20390074 and 17390071) and a grant-in-aid for exploratory research (16650097) from the Japan Society for the Promotion of Science, Tokyo, Japan, and by grants from the Daiichi Sankyo Pharmaceutical Co, Tokyo, Japan, the Research Foundation for Treatment of Metabolic Abnormalities, Osaka, Japan, the Novartis Foundation for the Promotion of Science, Tokyo, Japan, the Japan Heart Foundation Grant for Research on Arteriosclerosis Update, Tokyo, Japan, and the University of Occupational and Environmental Health for Advanced Research, Japan.

Disclosure

None declared.

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