Important Roles of Endothelium-Dependent Hyperpolarization in Coronary Microcirculation and Cardiac Diastolic Function in Mice

Yosuke Ikumi, MD, PhD, Takashi Shiroto, MD, PhD,* Shigeo Godo, MD, PhD,* Hiroki Saito, MD, PhD,* Shuhei Tanaka, MD, PhD,* Akiyo Ito, MD, PhD,* Shoko Kajitani, MD,* Yuto Monma, MD,* Satoshi Miyata, PhD,† Masato Tsutsui, MD, PhD,‡ and Hiroaki Shimokawa, MD, PhD*†*

Abstract: Endothelium-dependent hyperpolarization (EDH) factor is one of endothelium-derived relaxing factors and plays important roles especially in microvessels. We have previously demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) is an EDH factor produced by all types of nitric oxide synthases (NOSs), including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS. Recent studies have suggested the association between coronary microvascular dysfunction and cardiac diastolic dysfunction. However, the role of EDH in this issue remains to be fully elucidated. We thus examined whether EDH plays an important role in coronary microcirculation and if so, whether endothelial dysfunction, especially impaired EDH, is involved in the pathogenesis of cardiac diastolic dysfunction in mice. Using a Langendorff-perfused heart experiment, we examined the increase in coronary flow in response to bradykinin in the presence of indomethacin and N^{ω}-nitro-L-arginine (EDH condition) in wild-type, eNOS-knockout (KO), and nNOS/eNOS-double-KO mice. Compared with wild-type mice, EDH-mediated relaxations were increased in eNOS-KO mice but were significantly reduced in n/eNOS-KO mice. Catalase, a specific H_2O_2 scavenger, markedly inhibited EDH-mediated relaxations in all 3 genotypes, indicating compensatory roles of nNOS-derived H_2O_2 as an EDH factor in coronary microcirculation. Although both eNOS-KO and n/eNOS-KO mice exhibited similar extents of cardiac morphological changes, only n/eNOS-KO mice exhibited cardiac diastolic dysfunction. The expression of oxidized protein kinase G I-α (PKGIα) in the heart was significantly increased in eNOS-KO mice compared with n/eNOS-KO mice. These results indicate that EDH/H_2O_2 plays important roles in maintaining coronary microcirculation and cardiac diastolic function through oxidative PKGIα activation.

Key Words: endothelium, endothelium-dependent hyperpolarization, coronary microcirculation, cardiac diastolic function, nitric oxide synthase

(*J Cardiovasc Pharmacol*™ 2020;75:31–40)

INTRODUCTION

The endothelium plays an important role in modulating vascular tone by synthesizing and releasing endothelium-derived relaxing factors (EDRFs), including prostaglandins [mainly prostacyclin (PGI_2)], nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors.[1] We have previously demonstrated that the contribution of EDRFs varies depending on the size of blood vessels; NO predominantly regulates the tone of large conduit vessels while the contribution of EDH increases as the vessel size decreases.[2,3] Although several candidates have been proposed as a nature of EDH factor, including epoxyeicosatrienoic acids (metabolites of arachidonic P450 epoxygenase pathway),[4,5] electrical communication through gap junctions,[6] and K^+ ions,[7] we have previously demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) is one of the EDH factors in mouse[8] and human mesenteric arteries[9] and porcine coronary microvessels.[10] Mechanistically, endothelium-derived H_2O_2 oxidizes and activates protein kinase G Iα (PKGIα) in vascular smooth muscle cells, causing hyperpolarization-mediated vascular smooth muscle cells relaxation and vasodilatation.[11,12] We also have demonstrated that all 3 types of NO synthase (NOS) [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)] are the sources of H_2O_2 as an EDH factor.[13] Thus, the endothelial NOSs system serves not only as the NO-generating system in conduit arteries but also as the EDH-generating system in microvessels, finely modulating blood pressure and organ perfusion.[1,8–10] We also have previously demonstrated that in coronary microcirculation, EDH is involved in metabolic vasodilation,[14] coronary autoregulation,[15] and cardioprotective effects against ischemia-reperfusion injury,[16] indicating its crucial roles in maintaining homeostasis of coronary microcirculation. However, the nature and the source of EDH in coronary microcirculation remain to be fully elucidated.

Recently, the prevalence of heart failure with left ventricular (LV) diastolic dysfunction has been increasing,

Received for publication July 7, 2019; accepted September 1, 2019.

From the *Department of Cardiovascular Medicine; and †Evidence-based Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; and ‡Department of Pharmacology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Japan.

Supported in part by a grant-in-aid for Scientific Research on Innovative Areas (Signaling Functions of Reactive Oxygen Species), and a grant-in-aid for Scientific Research 16K19383, both of which are from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan.

The authors report no conflicts of interest.

Reprints: Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan (e-mail: shimo@cardio.med.tohoku.ac.jp).

which is recognized as an important pathological condition in heart failure.[17] Although the pathogenesis and molecular mechanism(s) of cardiac diastolic dysfunction remain to be fully clarified, Dirk et al reported an association between coronary microvascular dysfunction and cardiac diastolic dysfunction.[18] Moreover, Eaton et al demonstrated that oxidative activation of PKGIα in myocardial cells is involved in maintaining cardiac diastolic function through regulation of calcium handling.[19] These reports led us to hypothesize that the endothelium-derived H_2O_2 in coronary microcirculation plays important roles in maintaining cardiac diastolic function. To test this hypothesis, we used wild-type (WT), eNOS-knock-out (eNOS-KO), and nNOS/eNOS- doubly-KO (n/eNOS-KO) mice to examine the contributions of eNOS and nNOS in endothelium-dependent relaxations in coronary microcirculation and cardiac diastolic function.

METHODS

Animals

This study was reviewed and approved by the Committee on Ethics of Animal Experiments of Tohoku University (#2018MdA-188 and #2018MdA-182), which was granted by the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. All experiments were conducted in 12- to 16-week-old male mice. eNOS-KO mice were originally provided by P. Huang (Harvard Medical School, Boston, MA) and were backcrossed to C57BL/6 mice more than 10 generations. We generated doubly-n/eNOS-KO mice by crossing eNOS-KO and nNOS-KO mice as previously reported.[20] Male C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan) as a WT control. All animals were cared for in accordance with the regulations and rules configured by the committee, fed normal chow, and maintained on a 12-hour light and dark cycle.

Langendorff Experiments

Langendorff experiments were performed as previously described.[21–23] Mice were pretreated intraperitoneally with heparin (500 units), and then, 10 minutes later, they were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The hearts were excised from the mice and then placed into ice-cold Krebs–Henseleit buffer (KHB) to be arrested. After all extraaortic tissues were removed, the ascending aorta was cannulated with a 21-gauge blunted needle and tied up with a thread. Then, the heart was retrogradely perfused at a constant flow of 2.0 mL/min by the Langendorff apparatus (Model IPH-W2; Primetech Corporation, Tokyo, Japan) with warmed KHB bubbled with 95% O_2 and 5% CO_2. Once the heart was set onto the apparatus, the heart was surrounded by a water-jacketed organ bath for maintaining a constant temperature. After a 10-minute stabilization period, the heart was paced at 400 bpm and then perfused at a constant pressure of 80 mm Hg. Coronary flow was continuously measured with a flowmeter using an ultrasonic flow probe (FLSC-01; Primetech Corporation) and analyzed by a computer-based analysis system in LabChart 7.0 software. Bradykinin (BK, 10^{-6} mol/L) and sodium nitroprusside (SNP, 10^{-5} mol/L) were used to evaluate endothelium-dependent and endothelium-independent vasodilator responses, respectively. Indomethacin (Indo, a cyclooxygenase inhibitor, 10^{-5} mol/L) and N^{ω}-nitro-L-arginine (L-NNA, a NOS inhibitor, 10^{-4} mol/L) were used for inhibition of vasodilator prostaglandins and NOSs, respectively. EDH-mediated responses were measured as the remaining coronary flow in the presence of both Indo and L-NNA.[24] Catalase (Cat, 750 U/mL) was used as a specific H_2O_2 scavenger.[24] Coronary flow rate was corrected by heart weight. Baseline coronary flow was defined as coronary flow rate before administration of vasodilator agonists. Increases in coronary flow were calculated as the difference between maximum coronary flow after administration of agonists and baseline coronary flow (Fig. 1A).

Echocardiography, Cardiac Catheterization, and Blood Pressure Measurement

We performed echocardiography using the Vevo 2100 Imaging System (Visualsonics, Toronto, Canada) as previously described.[25] Mice were anesthetized with inhaled isoflurane and laid on a warmed table during the measurement. LV dimensions and systolic functions were measured in M-mode at the level of papillary muscles. Doppler signals of mitral inflow velocity and mitral annular velocity were obtained to calculate E/A and E/e' for assessment of cardiac diastolic function. By adjusting the concentration of isoflurane (0.5%–1.0%), heart rate was maintained at approximately 450–500 bpm. For LV pressure–volume relationship, the catheter (Millar Instruments, Houston, TX) was inserted into the LV through apical approach after the mice were intubated and received ventilation.[25] Blood pressure was measured by the tail-cuff system (MK-2000ST NP-NIBP; Muromachi Kikai Co, Ltd, Tokyo, Japan) without anesthesia.[25]

Western Blot Analysis

We performed Western blot analysis as previously described.[26] Whole hearts were isolated and snap frozen. The frozen hearts were homogenized with both tissue protein extract reagent (T-PER; Thermo Fisher Scientific, Rockford, MA) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation, the supernatants of the lysates were reconstituted in nonreducing sample buffer with maleimide (100 mmol/L) for PKGIα[12] and in reducing sample buffer without maleimide for others. They were then loaded on SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Fairfield county, CT). The membranes were blocked for 1 hour at room temperature. The primary antibodies used were as follows: PKGIα [PKGIα (1:500), Santa Cruz, #sc-393987], phospholamban [PLN (1:5000), Thermo Fisher Scientific, #MA3-922], pSer[16]-PLN (1:1000, Abcam, Cambridge, UK, #ab15000), sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a [SERCA2a (1:1000), Thermo Fisher Scientific, #2A7-A1], and glyceraldehyde-3-phosphate dehydrogenase [GAPDH (1:5000), Cell Signaling Technology, #2118]. The regions containing proteins were visualized by the enhanced chemiluminescence system (ECL Prime Western

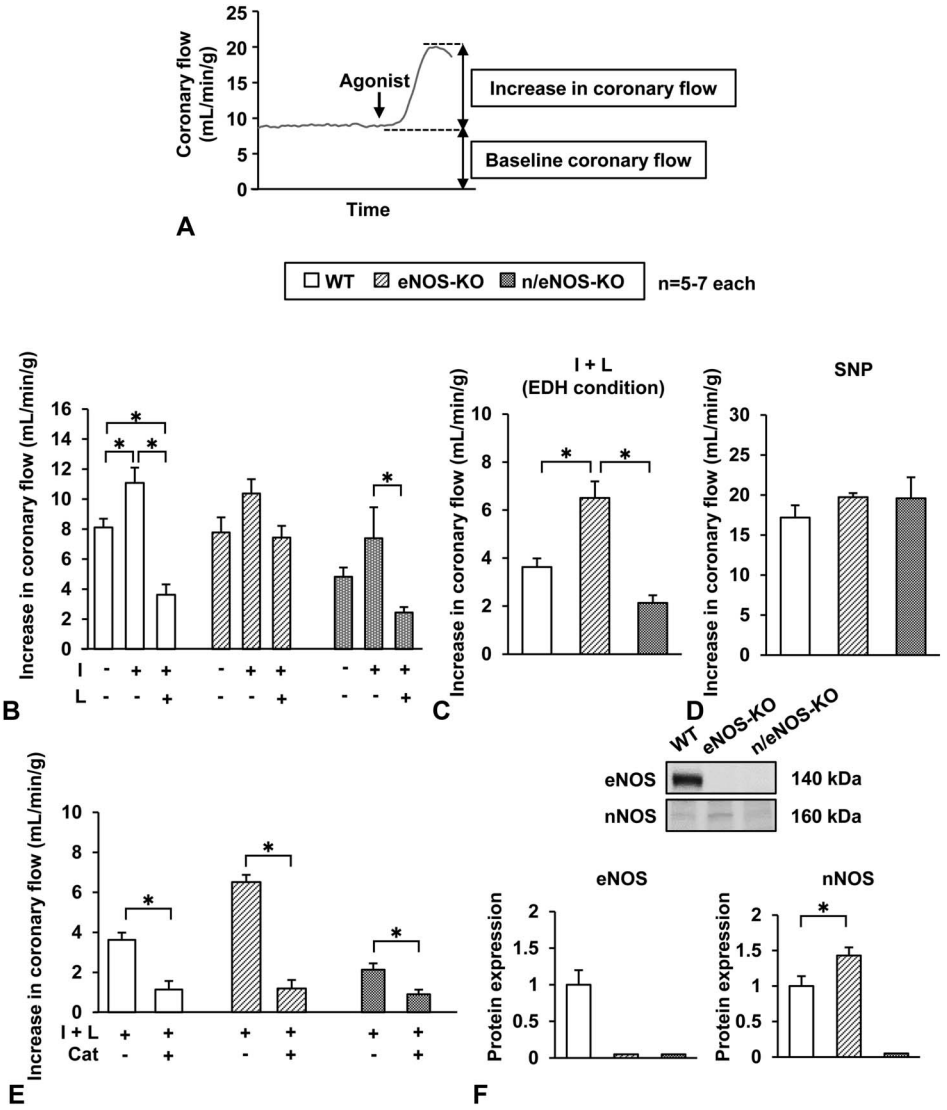

FIGURE 1. Endothelium-dependent and endothelium-independent relaxations in coronary microcirculation in mice. A, Representative recording in perfused heart experiment. Baseline coronary flow was defined as coronary flow before administration of vasodilator agonist. Increase in coronary flow was calculated as difference between maximum coronary flow after administration of agonist and baseline coronary flow. Coronary flows were corrected by heart weight. B, Increase in coronary flow to bradykinin with or without inhibitors including indomethacin (I) and L-NNA (L) in WT mice, eNOS-KO mice, and n/eNOS-KO mice (n = 7 in each condition in each mice). All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and are shown as mean ± SEM. $*P < 0.05$. C, Comparison in EDH-mediated relaxations among WT, eNOS-KO, and n/eNOS-KO mice (n = 7 each). All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and are shown as mean ± SEM. $*P < 0.05$. D, Endothelium-independent relaxation to sodium nitroprusside (SNP, 10^{-5} mol/L) in WT (n = 7), eNOS-KO (n = 7), and n/eNOS-KO mice (n = 6). All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and are shown as mean ± SEM. E, Comparisons between BK-mediated relaxations in EDH condition (I + L) with or without catalase (Cat; 750 U/mL) in WT, eNOS-KO, and n/eNOS-KO mice (n = 7 and 5, respectively for each mice). All results were analyzed by unpaired Student's *t*-test and are shown as mean ± SEM. $*P < 0.05$. F, Protein expression of eNOS and nNOS in the heart. Western blot analysis was performed for analysis of eNOS expression, while both immunoprecipitation and Western blots for analysis of nNOS expression (n = 7 each). Results were analyzed by unpaired Student's *t*-test and are shown as mean ± SEM.

Blotting Detection Reagent, GE Healthcare). Densitometric analysis was performed with the Image J Software (National Institutes of Health (NIH), Bethesda, MD).

Immunoprecipitation

Hearts were isolated and lysed in T-PER (Thermo Fisher Scientific), followed by centrifugation. The supernatants were adjusted equally at 300 μg of protein after BCA assay (BCA Protein Assay Kit; Thermo Fisher Scientific) and then incubated with anti-nNOS antibody (1:100; Thermo Fisher Scientific, #61-7000) for 1 hour at 4°C. After the incubation, 25 μL of pre-washed EZ view protein G affinity gel (Sigma-Aldrich) was added to the lysates, followed by 1-hour incubation at 4°C. After centrifugation, the supernatants were removed. A total of 50 mL of sample buffer (10% sodium dodecyl sulfate, 30% 2-mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue) was added and then heated at 95°C for 5 minutes. After centrifugation, the supernatants were analyzed by SDS/polyacrylamide gel electrophoresis and immunoblotting with anti-nNOS antibody (1:300; Thermo Fisher Scientific, #61-7000) for measurement of nNOS expression.

Histological Analysis

For histological analysis, mice were perfused with ice-cold KHB at physiological pressure. Then, excised hearts were fixed with 10% formaldehyde solution on a shaker at room temperature for 24 hours. After serial steps of washing and dehydration, the hearts were embedded in paraffin and sliced at 3 μm for making sections. The sections were stained with hematoxylin-eosin (HE) and Masson-trichrome (MT) or were used for immunostaining

with anti-CD31 antibody (1:400, Abcam). For analysis of myocardial cross-sectional area (CSA), images of HE-stained sections were obtained by computer-assisted imaging system (BX51; Olympus, Tokyo, Japan), and analyzed by tracing the outlines of cardiomyocyte with a clear nucleus image from the LV using the Image J Software. For quantifications of fibrosis area and capillary density, images were obtained using BZ-9000 series All-in-One Fluorescence Microscope (KEYENCE, Osaka, Japan) and analyzed using Hybrid Cell Count program of BZ-X analysis application (KEYENCE).

Reagents

SNP was purchased from Maruishi Seiyaku (Osaka, Japan), and all other drugs were from Sigma Aldrich (St. Lois, MO). The ionic composition of KHB was as follows (mmol/L); Na^+ 144, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 24, Cl^- 129.7, and glucose 5.5.

Statistical Analysis

All results are expressed as mean ± SEM. All comparisons of mean values were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or unpaired Student's *t*-test. Statistical analysis was performed in GraphPad Prism version 7.00 (GraphPad software, La Jolla, CA). Results were considered statistically significant at $P < 0.05$.

RESULTS

Compensatory Role of nNOS-Derived EDH/H_2O_2 in Maintaining Coronary Microcirculation in eNOS-KO Mice

To assess endothelium-dependent relaxations in coronary microcirculation, we measured BK-induced increases in coronary flow in the absence or presence of EDRF inhibitors (Fig. 1B). Specifically, to examine the differences in EDH responses among the 3 genotypes, we compared the increase in coronary flow with BK in the presence of both indomethacin and L-NNA (EDH condition) (Fig. 1C). EDH-mediated responses were significantly increased in eNOS-KO mice as compared with WT mice. Furthermore, the EDH-mediated response in n/eNOS-KO mice was significantly decreased as compared with eNOS-KO mice. These results indicate that nNOS-derived EDH plays a compensatory role in maintaining coronary flow in eNOS-KO mice. By contrast, SNP-mediated responses were comparable among the 3 genotypes (Fig. 1D), indicating that there was no difference in endothelium-independent relaxation among these mice. To further investigate whether H_2O_2 plays a role as an EDH factor in coronary microcirculation in mice, we examined the increase in coronary flow to BK with or without catalase (a specific H_2O_2 scavenger) in EDH condition. Catalase significantly and markedly inhibited the EDH responses in all 3 genotypes (Fig. 1E). These results indicate that H_2O_2 plays an important role in coronary microcirculation as an EDH factor. In Western blot analysis, the extent of nNOS in the heart was increased in eNOS-KO mice as compared with WT mice (Fig. 1F). These results indicate that nNOS-derived EDH/H_2O_2 plays a compensatory role in maintaining coronary flow in eNOS-KO mice.

Hypertension and Cardiac Hypertrophy in eNOS-KO and n/eNOS-KO Mice

In both eNOS-KO and n/eNOS-KO mice, systolic and diastolic blood pressures were significantly and similarly elevated compared with WT mice, with no difference between the 2 genotypes (Fig. 2A). Similarly, in eNOS-KO and n/eNOS-KO mice, as compared with WT mice, the ratio of heart/body weight was also significantly

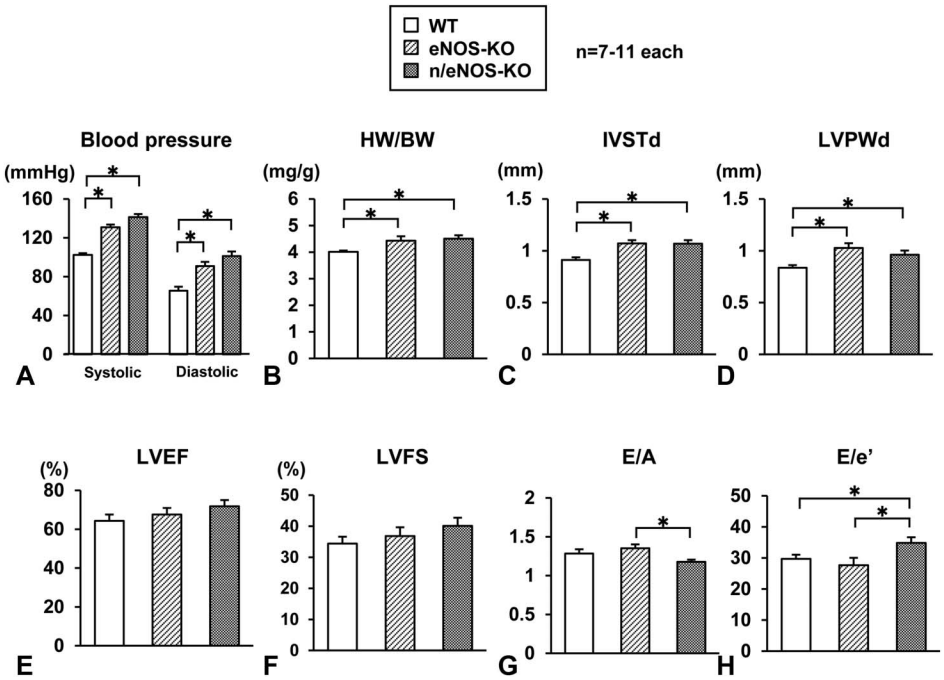

FIGURE 2. Blood pressures and cardiac phenotypes. A, Systolic and diastolic pressures measured with tail-cuff method (n = 7 each). B, Heart weight (HW)/body weight (BW) ratio (n = 7 each). C–H, Cardiac phenotypes assessed by echocardiography (n = 11 each). These data were obtained at 450–500 bpm. All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and are shown as mean ± SEM. *$P < 0.05$.

higher (Fig. 2B), and LV wall thickness measured by echocardiography was significantly increased (Figs. 2C, D). These results indicate that eNOS-KO and n/eNOS-KO mice exhibit similar extents of systemic hypertension and cardiac hypertrophy.

Cardiac Diastolic Dysfunction in n/eNOS-KO but Not in eNOS-KO Mice

Echocardiographic study showed that LV systolic functions, as assessed by LVEF and LVFS, were comparable among the 3 genotypes (Figs. 2E, F). By contrast, although cardiac diastolic functions, as assessed by E/A and E/e', were comparable between WT and eNOS-KO mice, the values of E/A were significantly reduced and those of E/e' were significantly elevated in n/eNOS-KO mice as compared with WT and eNOS-KO mice (Figs. 2G, H), indicating significant cardiac diastolic dysfunction in n/eNOS-KO mice. We also performed pressure–volume loop catheterization to assess the cardiac functions in more detail (Fig. 3). Consistent with the results of echocardiography, peak rate of fall in pressure (dp/dt min) was significantly decreased, and tau and end diastolic pressure–volume relationship (EDPVR) were significantly increased in n/eNOS-KO mice compared with WT mice (Figs. 3C–F), although LVEDP was comparable among the 3 genotypes. These results indicate that cardiac diastolic dysfunction was developed in n/eNOS-KO mice, but not in eNOS-KO mice, suggesting that nNOS compensates cardiac diastolic function in eNOS-KO condition.

Cardiac Morphological Changes in eNOS-KO and n/eNOS-KO Mice

To examine whether morphological changes in myocardial structure cause the difference in cardiac diastolic function between eNOS-KO and n/eNOS-KO mice, we performed histological analysis with myocardial tissue (Fig. 4A). Consistent with the findings for cardiac hypertrophy, myocardial CSA was significantly larger to the same extent in eNOS-KO and n/eNOS-KO mice compared with WT mice (Fig. 4B). Similarly, the extent of myocardial fibrosis was significantly higher to the same extent in eNOS-KO and n/eNOS-KO mice compared with WT mice (Fig. 4C). Also, capillary density was significantly reduced in eNOS-KO and n/eNOS-KO mice to the same extent as compared with WT mice (Fig. 4D). These results indicate that eNOS-KO and n/eNOS-KO mice exhibit similar extent of cardiac morphological changes.

Enhanced Oxidative Activation of PKGIα and Increased Phosphorylation of Phospholamban in the Heart of eNOS-KO Mice

Western blot analysis using whole heart lysates showed that dimeric PKGIα in eNOS-KO mice was significantly upregulated compared with n/eNOS-KO mice (Figs. 5A, B). Importantly, phosphorylation of PLN at Ser^{16} in eNOS-KO mice was also significantly increased compared with n/eNOS-KO mice (Fig. 5C), whereas total PLN and SERCA2a were

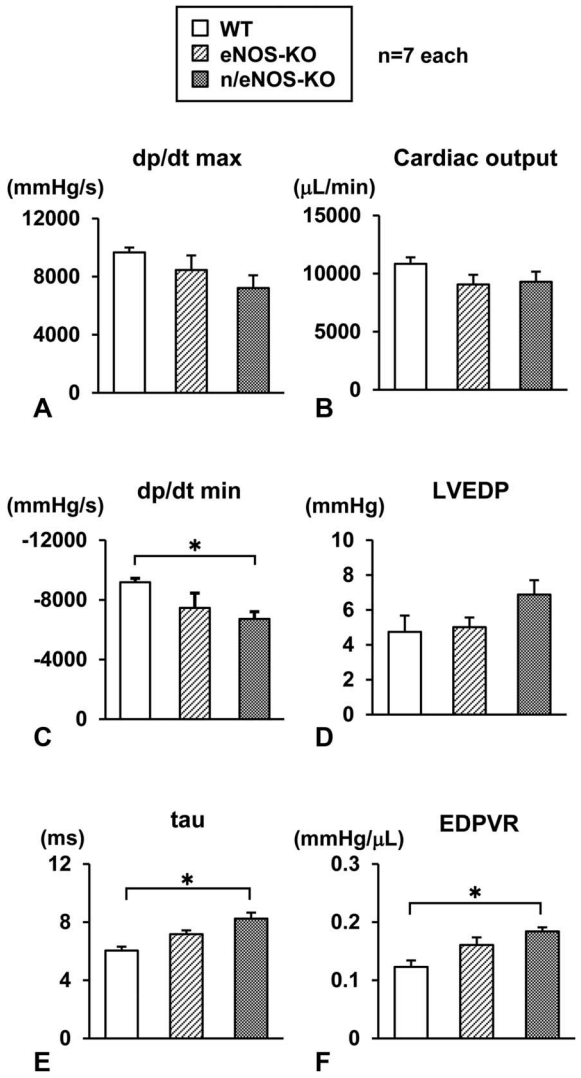

FIGURE 3. Pressure–volume loop analysis. Parameters obtained from pressure–volume loop catheterization (n = 7 each). A, Maximum rate of pressure rise (dp/dt max). B, Cardiac output. C, Peak rate of fall in pressure (dp/dt min). D, LV end diastolic pressure (LVEDP). E, Time constant (tau). F, End-diastolic pressure–volume relationship (EDPVR). All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and are shown as mean ± SEM. $*P < 0.05$.

comparable among the 3 genotypes (Figs. 5D, E). These results indicate that the enhanced oxidative activation of PKGIα may be associated with preserved cardiac diastolic function in eNOS-KO mice through PLN activation, in which compensatory and enhanced nNOS-derived EDH/H_2O_2 in coronary microcirculation in eNOS-KO mice may be involved in this oxidative pathway.

DISCUSSION

The major findings of this study are as follows: (1) EDH plays an important role in endothelium-dependent relaxations in

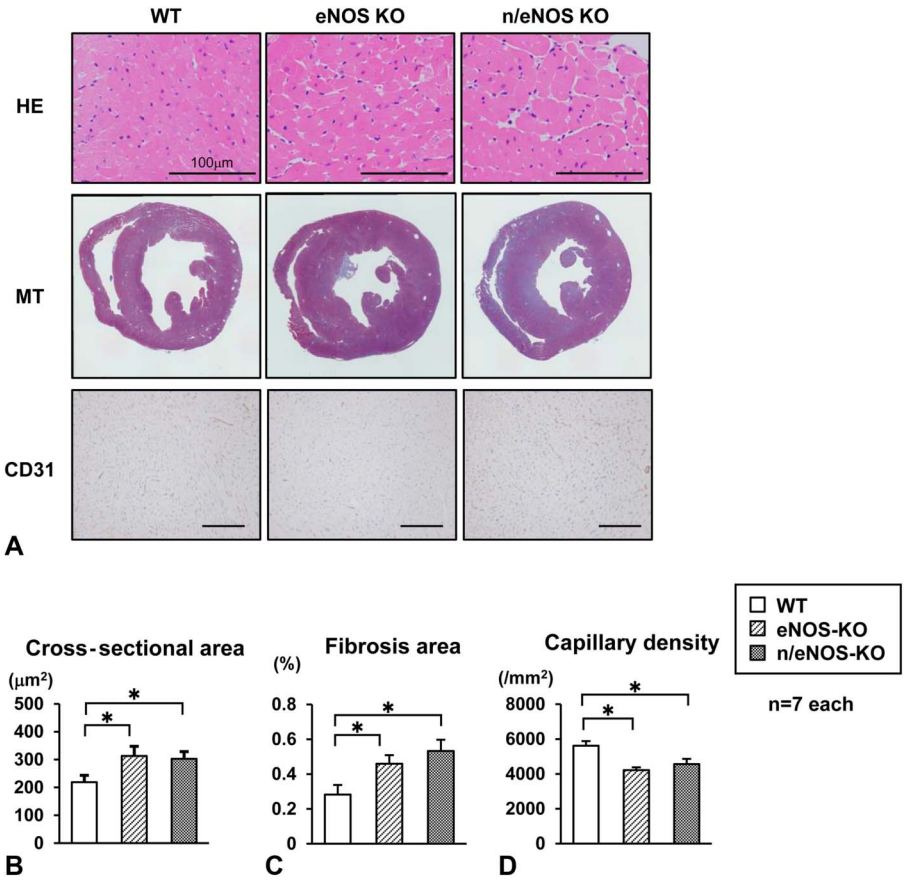

FIGURE 4. Histological analysis of myocardial tissue. A, Representative images of myocardial tissues stained with hematoxylin-eosin (HE, upper), Masson trichrome (MT, middle), and immunostaining for anti-CD31 (lower). Scale bars = 100 μm. B, Quantitative analysis of CSA obtained from HE-stained sections (n = 7 each). C, Quantitative analysis of myocardial fibrosis area in the images of MT-stained sections (n = 7 each). D, Quantitative analysis of capillary density in the images of CD31 immunostaining (n = 7 each). All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and are shown as mean ± SEM. $*P < 0.05$.

coronary microcirculation in mice, (2) endothelium-derived H_2O_2 plays an important role as an EDH factor in coronary microcirculation in mice, (3) nNOS-derived EDH/H_2O_2 is enhanced in coronary microcirculation in eNOS-KO mice, (4) cardiac diastolic dysfunction is noted in n/eNOS-KO mice but not in eNOS-KO mice, although there are no remarkable differences in blood pressure or myocardial morphology between the 2 genotypes, and (5) nNOS-derived EDH/H_2O_2 oxidizes PKGIα in the hearts, preserving cardiac diastolic function in eNOS-KO mice. To the best of our knowledge, this is the first study that demonstrates that EDH/H_2O_2 plays important roles in maintaining coronary microcirculation and cardiac diastolic function through oxidative PKGIα activation (Fig. 6).

Compensatory Roles of nNOS-Derived EDH/H_2O_2 in eNOS-KO Mice

This study demonstrated that EDH/H_2O_2 plays an important role in maintaining homeostasis of coronary microcirculation, and that nNOS-derived EDH/H_2O_2 plays compensatory roles in eNOS-KO mice. It is known that eNOS generates superoxide anion (O_2^-) from its reductase domain under normal conditions, whereas it also generates O_2^- from its oxidase domain only when pathologically uncoupled [eg, tetrahydrobiopterin (BH_4) and/or L-arginine depletion], and that L-arginine analogues only inhibit the latter process.[27] We have previously demonstrated that H_2O_2 is an EDH factor generated from the reductase domain of eNOS, which process is unaffected by L-arginine analogues, and that EDH-mediated relaxations are markedly reduced in eNOS-KO mice, where the remaining 2 NOSs (nNOS and iNOS) play compensatory roles for EDH responses.[13] Interestingly, in this study, we demonstrated that EDH-mediated relaxations in coronary microcirculation were enhanced in eNOS-KO mice compared with WT mice and were significantly reduced in n/eNOS-KO mice. It is possible that the difference in the contributions of eNOS and nNOS as EDH-generating systems between the present and the previous studies is attributed to the difference in vascular beds tested. Several studies reported that nNOS plays a compensatory role in coronary microvessels in eNOS-KO mice, and that EDH may be involved in this compensatory mechanism,[28,29] which findings are consistent with this study. All these findings indicate that nNOS-dependent EDH plays an important compensatory role in maintaining coronary flow in eNOS-deficient condition. In general, NO-mediated relaxations are impaired in hypertension, diabetes mellitus, and dyslipidemia because of the decreased eNOS-derived NO bioavailability.[18,30–32] Thus, in the pathological conditions with eNOS dysfunction caused by those risk factors, the compensatory role of nNOS-derived EDH/H_2O_2 may be more important than in control condition in maintaining coronary flow. However,

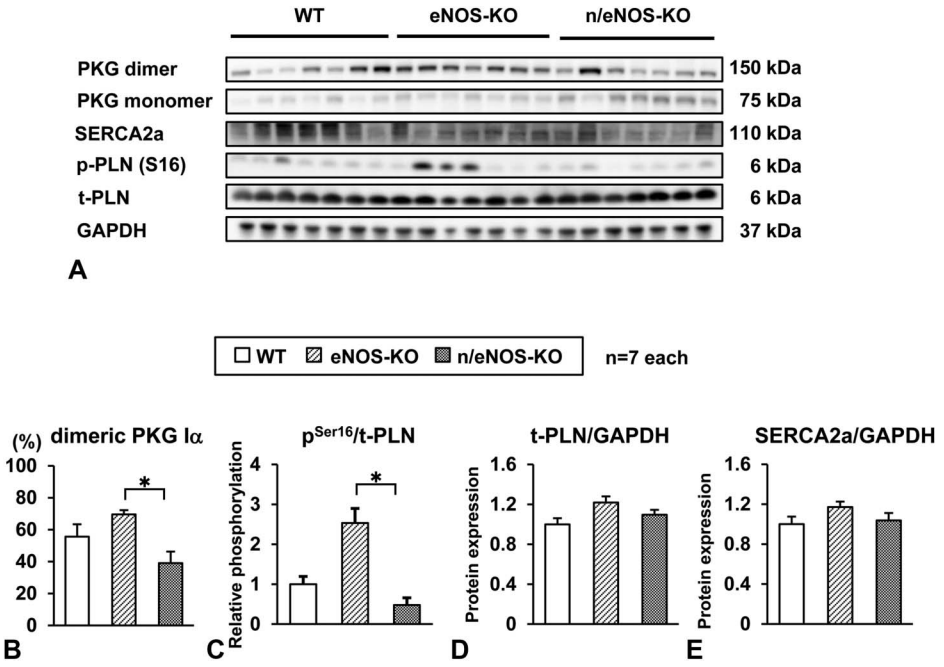

FIGURE 5. Mechanisms of maintained cardiac diastolic function in eNOS-KO mice. Western blot analyses were performed using whole hearts (n = 7 each). A, Western blots for PKGIα, pSer16-phospholamban (PLN), total PLN, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, Quantitative analysis of PKG dimerization defined as the ratio of dimeric PKG to both dimeric and monomeric PKG. C, Quantitative analysis of phosphorylated PLN (Ser16) as normalized to total protein expressions. D, Quantitative analysis of total PLN as normalized to GAPDH. E, Quantitative analysis of SERCA2a as normalized to GAPDH. All results were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons are shown as mean ± SEM. $*P < 0.05$.

we have previously demonstrated that nitrative stress caused by excessive NO leads to desensitization of blood vessels to EDH/H_2O_2, cardiac hypertrophy, and impaired coronary microcirculation in mice.[29] Moreover, a recent study also indicates that nitrosative stress causes cardiac diastolic dysfunction.[33] These reports indicate that excessive NO plays a harmful role in cardiovascular system in mice. Therefore, it is possible that nNOS-derived EDH rather than NO is enhanced in coronary microcirculation when eNOS functions are impaired. Thus, nNOS-derived EDH/H_2O_2 could be a new therapeutic target for cardiovascular diseases with impaired eNOS functions and reduced NO bioavailability.

Cardiac Diastolic Functions, EDH/H_2O_2, and PKGIα

This study demonstrates the association between cardiac diastolic function and EDH/H_2O_2 in coronary microcirculation. Recently, several studies suggested that coronary microvascular dysfunction plays a pivotal role in the pathogenesis of cardiac diastolic dysfunction.[18,34,35] Although the mechanisms are not fully elucidated, it is widely known that inflammation and oxidative stress in the coronary microvasculature associated with coronary risk factors (eg, hypertension, diabetes mellitus, and dyslipidemia) have been pointed out as major etiologies.[18,36] In the pathological conditions described above, TNF-α and IL-6 cause inflammation, leading to coronary microvascular dysfunction.[36] Subsequently, ROS derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase due to inflammation directly injures myocardium and reduces NO bioavailability, inhibiting the NO/cGMP/PKG pathway, an important modulator of cardiac diastolic function with resultant cardiac diastolic dysfunction.[18,36] A recent study also demonstrated that chronic and excessive H_2O_2 induces severe cardiac systolic dysfunction.[37] Thus, ROS (such as O_2^- and H_2O_2 in pathologically high concentrations) is usually considered harmful for the cardiovascular system. However, this study clearly indicates that EDH/H_2O_2 plays an important role in maintaining coronary microcirculation and cardiac diastolic function, suggesting that ROS in physiological concentrations plays important roles in maintaining cardiovascular homeostasis. Recently, Scotcher et al[19] reported that oxidative activation of PKGIα caused by ROS is one of the important mechanisms for maintaining cardiac diastolic function. In this study, Western blot analysis using the heart lysates showed that oxidative PKGIα (ie, dimeric status) was increased in eNOS-KO mice associated with enhanced EDH-mediated relaxations, while oxidized PKGIα was reduced in n/eNOS-KO mice associated with reduced EDH-mediated relaxations. The present results of echocardiography and pressure–volume loop catheterization showed that cardiac diastolic dysfunction was noted in n/eNOS-KO mice but not in eNOS-KO mice, a consistent finding with the recent report by Scotcher et al.[19] Taken together, this study indicates that EDH/H_2O_2 is substantially involved in maintaining cardiac diastolic function through oxidative activation of PKGIα.

No Differences in Cardiac Morphological Changes Between eNOS-KO and n/eNOS-KO Mice

In this study, histopathological examination showed no remarkable differences between eNOS-KO and n/eNOS-KO mice, whereas cardiac diastolic dysfunction was noted only in n/eNOS-KO. Although reduced capillary density, enhanced myocardial fibrosis, and cardiac hypertrophy are involved in the pathogenesis of cardiac diastolic dysfunction,[38] the difference in cardiac diastolic function between the 2 genotypes may be attributed to the changes in the signaling pathway rather than the changes in myocardial morphology. Oxidized PKGIα specifically phosphorylates PLN at Ser,[16]

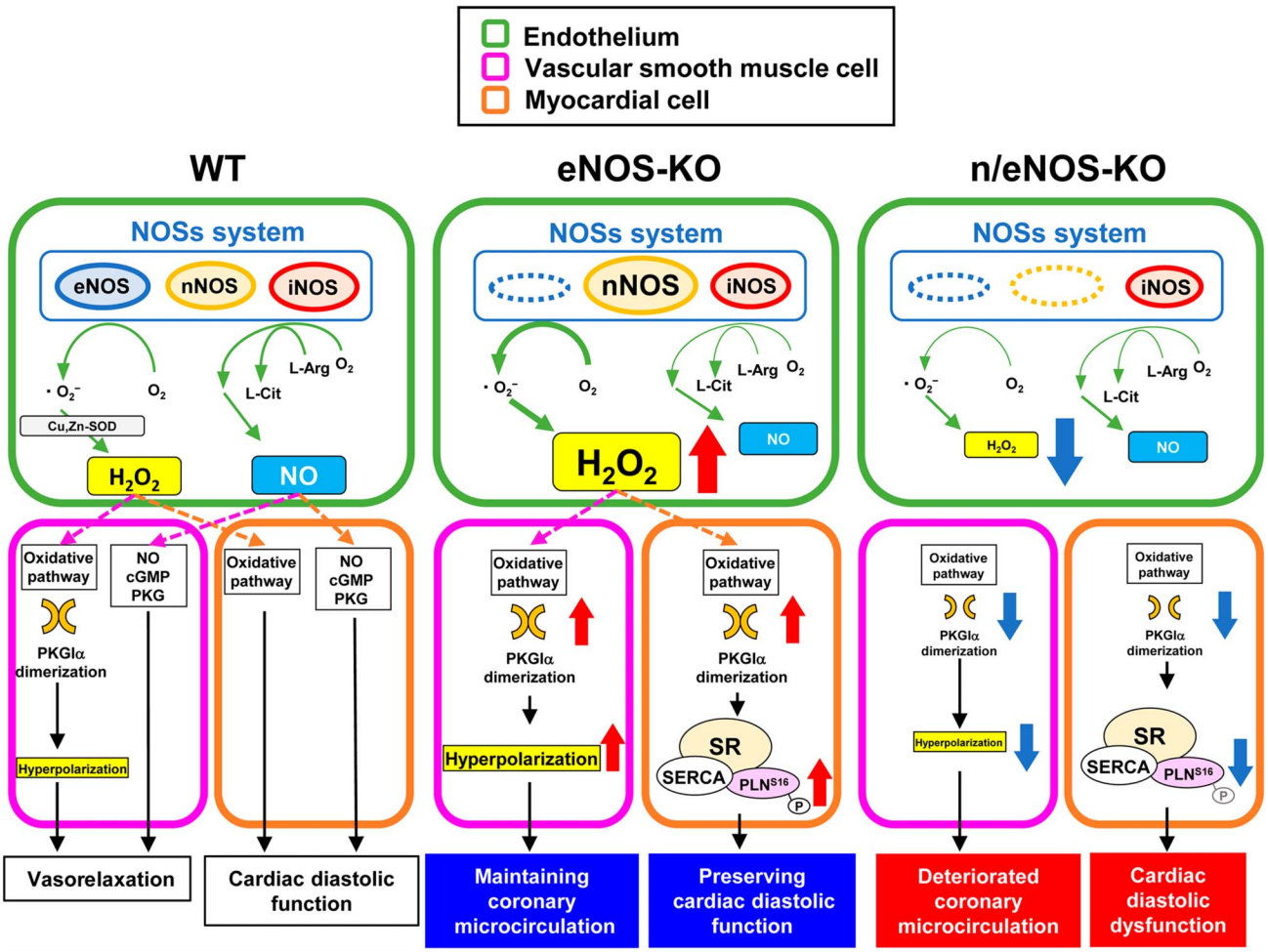

FIGURE 6. Summary of the present findings. (Left) In the coronary microcirculation of WT mice, endothelium-derived H_2O_2 plays important roles in maintaining cardiovascular homeostasis and cardiac diastolic function. (Middle) In eNOS-KO mice, nNOS-derived H_2O_2 is increased and plays compensatory roles in maintaining coronary microcirculation through enhancing EDH in vascular smooth muscle cells and in preserving cardiac diastolic function by phosphorylating PLN at Ser16 in myocardial cells through oxidative PKGIα activation. (Right) In e/nNOS-KO mice, compensatory roles of nNOS are absent, resulting in coronary microcirculatory dysfunction and cardiac diastolic dysfunction. Cu,Zn-SOD, copper, zinc-superoxide dismutase; L-Arg,L-arginine; L-Cit,L-citrulline; SR, sarcoplasmic reticulum.

leading to disinhibition of SERCA2a and activation of calcium handling in myocardial cells to enhance cardiac diastolic function.[19] Indeed, this study showed that phosphorylation of PLN at Ser^{16} was increased in eNOS-KO mice compared with n/eNOS-KO mice. These results are consistent with the mechanisms mentioned above and might be attributed to the oxidative activation of PKGIα by compensatory nNOS-derived EDH/H_2O_2 in coronary microcirculation. Thus, EDH/H_2O_2 could be a novel therapeutic target for cardiac diastolic dysfunction even when the myocardial remodeling has already advanced and is irreversible.

Of note, previous studies demonstrated that PKA-dependent phosphorylation of PLN and intracellular Ca^{2+} decay by nNOS-derived NO were impaired in cardiomyocytes isolated from nNOS-KO mice, causing the impairments of myocardial relaxation and cardiac diastolic function in nNOS-KO mice.[39,40] Importantly, however, EDH/H_2O_2-mediated vasorelaxation was decreased by single knockdown of nNOS.[41] Assuming that EDH/H_2O_2 in coronary microcirculation in nNOS-KO mice is decreased, it is possible that cardiac diastolic dysfunction in nNOS-KO mice could be attributed not only to impaired NO-mediated signaling in cardiomyocytes but also to altered EDH/H_2O_2-mediated responses in coronary microvessels. Thus, further studies are needed to clarify the contribution of EDH/H_2O_2 in cardiac diastolic function in nNOS-KO mice.

Study Limitations

Several limitations should be mentioned for this study. First, nNOS-KO mice were not tested in this study as already mentioned. Considering that EDH/H_2O_2-mediated vasorelaxation was decreased by nNOS knockdown,[41] EDH/H_2O_2-mediated relaxation in coronary microcirculation may be decreased in nNOS-KO mice, possibly

contributing to cardiac diastolic dysfunction. Second, in addition to eNOS and nNOS, iNOS also could serve as EDH-generating system in mice.[13] Because we used only eNOS-KO and n/eNOS-KO mice in this study, the roles of iNOS in coronary microcirculation remain to be examined in future studies. Third, the negative interaction between NO and EDH was not examined in this study because of technical limitations of Langendorff experiment. Previous studies showed that NO donors attenuate EDH-mediated responses in coronary arteries.[42–44] Thus, the increase in EDH in coronary microcirculation in eNOS-KO mice could result from the decrease in NO derived from eNOS. Further studies are needed to clarify this issue. Fourth, the cause and effect relationships among oxidative activation of PKGIα, myocardial morphological changes, and diastolic function remain to be examined in future studies. In this study, because the mice used in each experiment were from different groups, we were unable to analyze these correlations. Fifth, it is known that several other proteins, such as titin,[45] ryanodine receptor,[46,47] and Rho-kinase,[25] are involved in cardiac diastolic functions. Thus, further studies are needed to examine whether these proteins play roles in compensation for cardiac diastolic function in addition to nNOS-derived EDH. Sixth, although the NO/cGMP/PKG pathway is known to be involved in modulating cardiac diastolic function,[48–50] we focused on the oxidative pathway of PKGIα in this study. Recent studies demonstrated that eNOS-KO mice do not exhibit cardiac diastolic dysfunction in normal condition, although eNOS is the major source of NO.[51,52] The reason why eNOS-KO mice exhibit preserved diastolic function could be explained, at least in part, by the compensatory role of nNOS-derived EDH/H_2O_2 as demonstrated in this study.

CONCLUSIONS

In this study, we were able to demonstrate that nNOS-derived EDH/H_2O_2 plays a crucial role in maintaining coronary microcirculation in eNOS-deficient condition and is substantially involved in preserving cardiac diastolic function through oxidative PKGIα activation. This study indicates that EDH/H_2O_2 in coronary microcirculation may be a possible novel therapeutic target for cardiac diastolic dysfunction and other cardiovascular diseases with impaired eNOS functions and reduced NO bioavailability.

ACKNOWLEDGMENTS

The authors appreciate S. Sato, Y. Watanabe, H. Yamashita, and A. Nishihara for excellent technical supports.

REFERENCES

1. Shimokawa H. 2014 Williams Harvey Lecture: importance of coronary vasomotion abnormalities-from bench to bedside. *Eur Heart J.* 2014;35:3180–3193.
2. Urakami-Harasawa L, Shimokawa H, Nakashima M, et al. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest.* 1997;10:2793–2799.
3. Shimokawa H, Yasutake H, Fujii K, et al. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol.* 1996;28:703–711.
4. Campbell WB, Gebremedhin D, Pratt PF, et al. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res.* 1996;78:415–423.
5. Fisslthaler B, Popp R, Kiss L, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature.* 1999;401:493–497.
6. Griffith TM, Chaytor AT, Edwards DH. The obligatory link: role of gap junctional communication in endothelium-dependent smooth muscle hyperpolarization. *Pharmacol Res.* 2004;49:551–564.
7. Edwards G, Dora KA, Gardener MJ, et al. K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature.* 1998;396:269–272.
8. Matoba T, Shimokawa H, Nakashima M, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest.* 2000;106:1521–1530.
9. Matoba T, Shimokawa H, Kubota H, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem Biophys Res Commun.* 2002;290:909–913.
10. Matoba T, Shimokawa H, Morikawa K, et al. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol.* 2003;23:1224–1230.
11. Burgoyne JR, Madhani M, Cuello F, et al. Cysteine redox sensor in PKGIα enables oxidant-induced activation. *Science.* 2007;317:1393–1397.
12. Prysyazhna O, Rudyk O, Eaton P. Single atom substitution in mouse protein kinase G eliminates oxidant sensing to cause hypertension. *Nat Med.* 2012;18:286–290.
13. Takaki A, Morikawa K, Tsutsui M, et al. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med.* 2008;205:2053–2063.
14. Yada T, Shimokawa H, Hiramatsu O, et al. Important role of endogenous hydrogen peroxide in pacing-induced metabolic coronary vasodilation in dogs in vivo. *J Am Coll Cardiol.* 2007;50:1272–1278.
15. Yada T, Shimokawa H, Hiramatsu O, et al. Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation.* 2003;107:1040–1045.
16. Yada T, Shimokawa H, Hiramatsu O, et al. Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo. *Am J Physiol Heart Circ Physiol.* 2006;291:H1138–H1146.
17. Shiba N, Nochioka K, Miura M, et al. Trend of westernization of etiology and clinical characteristics of heart failure patients in Japan—first report from the CHART-2 study. *Circ J.* 2011;75:823–833.
18. Sorop O, Heinonen I, van Kranenburg M, et al. Multiple common comorbidities produce left ventricular diastolic dysfunction associated with coronary microvascular dysfunction, oxidative stress, and myocardial stiffening. *Cardiovasc Res.* 2018;114:954–964.
19. Scotcher J, Prysyazhna O, Boguslavskyi A, et al. Disulfide-activated protein kinase G Iα regulates cardiac diastolic relaxation and fine-tunes the Frank-Starling response. *Nat Commun.* 2016;7:13187.
20. Morishita T, Tsutsui M, Shimokawa H, et al. Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. *Proc Natl Acad Sci U S A.* 2005;102:10616–10621.
21. Enkhjargal B, Godo S, Sawada A, et al. Endothelial AMP-activated protein kinase regulates blood pressure and coronary flow responses through hyperpolarization mechanism in mice. *Arterioscler Thromb Vasc Biol.* 2014;34:1505–1513.
22. Godo S, Sawada A, Saito H, et al. Disruption of physiological balance between nitric oxide and endothelium-dependent hyperpolarization impairs cardiovascular homeostasis in mice. *Arterioscler Thromb Vasc Biol.* 2016;36:97–107.
23. Saito H, Godo S, Sato S, et al. Important role of endothelial caveolin-1 in the protective role of endothelium-dependent hyperpolarization against nitric oxide-mediated nitrative stress in microcirculation in mice. *J Cardiovasc Pharmacol.* 2018;71:113–126.
24. Morikawa K, Shimokawa H, Matoba T, et al. Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest.* 2003;112:1871–1879.
25. Sunamura S, Satoh K, Kurosawa R, et al. Different roles of myocardial ROCK1 and ROCK2 in cardiac dysfunction and postcapillary pulmonary hypertension in mice. *Proc Natl Acad Sci U S A.* 2018;115:E7129–E7138.

26. Tanaka S, Shiroto T, Godo S, et al. Important role of endothelium-dependent hyperpolarization in pulmonary microcirculation in male mice -Implications for hypoxia-induced pulmonary hypertension. *Am J Physiol Heart Circ Physiol.* 2018;314:H940–H953.
27. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *J Biol Chem.* 2001;276:14533–14536.
28. Lamping KG, Nuno DW, Shesely EG, et al. Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice. *Am J Physiol Heart Circ Physiol.* 2000;279:H1906–H1912.
29. Talukder MA, Yang F, Shimokawa H, et al. eNOS is required for acute in vivo ischemic preconditioning of the heart: effects of ischemic duration and sex. *Am J Physiol Heart Circ Physiol.* 2010;299:H437–H445.
30. De Vriese AS, Verbeuren TJ, Van de Voorde J, et al. Endothelial dysfunction in diabetes. *Br J Pharmacol.* 2000;130:963–974.
31. Laursen JB, Somers M, Kurz S, et al. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation.* 2001;103:1282–1288.
32. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J.* 2012;33:829–837.
33. Schiattarella GG, Altamirano F, Tong D, et al. Nitrosative stress drives heart failure with preserved ejection fraction. *Nature.* 2019;568:351–356.
34. Hamdani N, Hervent AS, Vandekerckhove L, et al. Left ventricular diastolic dysfunction and myocardial stiffness in diabetic mice is attenuated by inhibition of dipeptidyl peptidase 4. *Cardiovasc Res.* 2014;104:423–431.
35. Crea F, Bairey Merz CN, Beltrame JF, et al. The parallel tales of microvascular angina and heart failure with preserved ejection fraction: a paradigm shift. *Eur Heart J.* 2017;38:473–477.
36. Paulus WJ, Tschope C. A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *J Am Coll Cardiol.* 2013;62:263–271.
37. Steinhorn B, Sorrentino A, Badole S, et al. Chemogenetic generation of hydrogen peroxide in the heart induces severe cardiac dysfunction. *Nat Commun.* 2018;9:4044.
38. Mohammed SF, Hussain S, Mirzoyev SA, et al. Coronary microvascular rarefaction and myocardial fibrosis in heart failure with preserved ejection fraction. *Circulation.* 2015;131:550–559.
39. Zhang YH, Zhang MH, Sears CE, et al. Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. *Circ Res.* 2008;102:242–249.
40. Dawson D, Lygate CA, Zhang MH, et al. nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. *Circulation.* 2005;112:3729–3737.
41. Capettini LS, Cortes SF, Gomes MA, et al. Neuronal nitric oxide synthase-derived hydrogen peroxide is a major endothelium-dependent relaxing factor. *Am J Physiol Heart Circ Physiol.* 2008;295:H2503–H2511.
42. Bauersachs J, Popp R, Hecker M, et al. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. *Circulation.* 1996;94:3341–3347.
43. Nishikawa Y, Stepp DW, Chilian WM. Nitric oxide exerts feedback inhibition on EDHF-induced coronary arteriolar dilation in vivo. *Am J Physiol Heart Circ Physiol.* 2000;279:H459–H465.
44. Olmos L, Mombouli JV, Illiano S, et al. cGMP mediates the desensitization to bradykinin in isolated canine coronary arteries. *Am J Physiol.* 1995;268:H865–H870.
45. Borlaug BA, Paulus WJ. Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment. *Eur Heart J.* 2011;32:670–679.
46. Kass DA, Bronzwaer JG, Paulus WJ. What mechanisms underlie diastolic dysfunction in heart failure? *Circ Res.* 2004;94:1533–1542.
47. Gonzalez DR, Treuer AV, Castellanos J, et al. Impaired S-nitrosylation of the ryanodine receptor caused by xanthine oxidase activity contributes to calcium leak in heart failure. *J Biol Chem.* 2010;285:28938–28945.
48. Shah AM, Spurgeon HA, Sollott SJ, et al. 8-bromo-cGMP reduces the myofilament response to Ca^{2+} in intact cardiac myocytes. *Circ Res.* 1994;74:970–978.
49. Heymes C, Vanderheyden M, Bronzwaer JG, et al. Endomyocardial nitric oxide synthase and left ventricular preload reserve in dilated cardiomyopathy. *Circulation.* 1999;99:3009–3016.
50. Kruger M, Kotter S, Grutzner A, et al. Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. *Circ Res.* 2009;104:87–94.
51. Gyurko R, Kuhlencordt P, Fishman MC, et al. Modulation of mouse cardiac function in vivo by eNOS and ANP. *Am J Physiol Heart Circ Physiol.* 2000;278:H971–H981.
52. van Deel ED, Octavia Y, de Boer M, et al. Normal and high eNOS levels are detrimental in both mild and severe cardiac pressure-overload. *J Mol Cell Cardiol.* 2015;88:145–154.