

Disruption of Physiological Balance Between Nitric Oxide and Endothelium-Dependent Hyperpolarization Impairs Cardiovascular Homeostasis in Mice

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Objective—Endothelium-derived nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) play important roles in modulating vascular tone in a distinct vessel size-dependent manner; NO plays a dominant role in conduit arteries and EDH in resistance vessels. We have recently demonstrated that endothelial NO synthase (eNOS) is functionally suppressed in resistance vessels through caveolin-1 (Cav-1)-dependent mechanism, switching its function from NO to EDH/hydrogen peroxide generation in mice. Here, we examined the possible importance of the physiological balance between NO and EDH in cardiovascular homeostasis.

Approach and Results—We used 2 genotypes of mice in which eNOS activity is genetically upregulated; Cav-1-knockout (Cav-1-KO) and endothelium-specific eNOS transgenic (eNOS-Tg) mice. Isometric tension recordings and Langendorff experiments with isolated perfused hearts showed that NO-mediated relaxations were significantly enhanced, whereas EDH-mediated relaxations were markedly reduced in microcirculations. Importantly, impaired EDH-mediated relaxations of small mesenteric arteries from Cav-1-KO mice were completely rescued by crossing the mice with those with endothelium-specific overexpression of Cav-1. Furthermore, both genotypes showed altered cardiovascular phenotypes, including cardiac hypertrophy in Cav-1-KO mice and hypotension in eNOS-Tg mice. Finally, we examined cardiac responses to chronic pressure overload by transverse aortic constriction in vivo. When compared with wild-type mice, both Cav-1-KO and eNOS-Tg mice exhibited reduced survival after transverse aortic constriction associated with accelerated left ventricular systolic dysfunction, reduced coronary flow reserve, and enhanced myocardial hypoxia.

Conclusions—These results indicate that excessive endothelium-derived NO with reduced EDH impairs cardiovascular homeostasis in mice in vivo. (*Arterioscler Thromb Vasc Biol.* 2016;36:97-107. DOI: 10.1161/ATVBAHA.115.306499.)

Key Words: caveolin-1 ■ endothelium ■ endothelium-dependent hyperpolarization factor ■ nitric oxide

The endothelium plays an important role in modulating vascular tone by synthesizing and releasing endothelium-derived relaxing factors, including vasodilator prostaglandins, nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors.¹⁻⁵ In 1988, Feletou and Vanhoutte⁶ and Chen et al⁷ independently demonstrated that a diffusible substance released by the endothelium causes relaxation and hyperpolarization of underlying vascular smooth muscle cells (VSMCs), attributing to the existence of putative EDH factors. A quarter century has passed since then and now several candidates have been proposed for the nature of EDH factors. It is widely accepted that the nature of EDH factors varies depending on species and vascular beds examined, including epoxyeicosatrienoic acids, metabolites of arachidonic P450 epoxygenase pathway,^{8,9} electric communication through gap junctions,¹⁰ K⁺ ions,¹¹ hydrogen sulfide,¹² and as we have originally identified¹³ and

other researchers have subsequently confirmed,¹⁴ endothelium-derived hydrogen peroxide (H₂O₂). Intriguingly, the contribution of endothelium-derived relaxing factors to endothelium-dependent vasodilatation markedly varies depending on vessel size with the physiological balance between NO and EDH; NO predominantly regulates the tone of large conduit vessels and the contribution of NO decreases as vessel size decreases, whereas that of EDH increases as vessel size decreases.^{15,16} Thus, EDH rather than NO plays a dominant role in small resistance vessels where blood pressure and organ perfusion are finely regulated. Indeed, accumulating evidence has demonstrated the critical roles of EDH in modulating blood pressure¹⁷ and vascular metabolic functions¹⁸ in general and coronary autoregulation¹⁹ and metabolic dilatation²⁰ in particular.

We have previously demonstrated the diverse roles of the NO synthases (NOSs) system in the endothelium depending

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Nonstandard Abbreviations and Acronyms

Cav-1	caveolin-1
Cav-1-KO	caveolin-1-knockout
EDH	endothelium-dependent hyperpolarization
eNOS	endothelial nitric oxide synthase
eNOS-Tg	endothelium-specific endothelial nitric oxide synthase transgenic
H₂O₂	hydrogen peroxide
L-NNA	N ^ω -nitro-L-arginine
NO	nitric oxide
TAC	transverse aortic constriction
VSMC	vascular smooth muscle cell

on blood vessel size.²¹ In large conduit vessels, NOS mainly serves as a NO-generating system to cause vasodilatation through soluble guanylate cyclase–cyclic guanosine monophosphate pathway. In contrast, in small resistance vessels, NOS serves as a superoxide-generating system to cause EDH-mediated responses through H₂O₂-induced protein kinase G1 α dimerization and subsequent activation of potassium channels, leading to hyperpolarization and vasodilatation.²² Furthermore, we have recently demonstrated that endothelial NOS (eNOS) is functionally inhibited in resistance vessels through caveolin-1 (Cav-1)-dependent mechanism, switching its function from NO-generating enzyme to EDH/H₂O₂-generating enzyme in mice.²³ This vessel size-dependent contribution of NO and EDH is well preserved from rodents to humans^{15,16}; however, the importance of the physiological balance between NO and EDH in cardiovascular homeostasis remains to be elucidated. In the clinical settings, it has been reported that chronic nitrate therapy could exert harmful effects in patients with myocardial infarction,²⁴ suggesting the importance of the physiological balance between NO and EDH/H₂O₂ for cardiovascular homeostasis.

In this study, we thus aimed to examine the possible importance of the physiological balance between NO and EDH in endothelium-dependent vasodilatation and cardiovascular homeostasis. To address this important issue, we used 2 genetically engineered mouse models with enhanced eNOS activity; systemic Cav-1-knockout (Cav-1-KO) mice,²⁵ and endothelium-specific eNOS transgenic (eNOS-Tg) mice.²⁶ We tested our hypothesis that excessive endothelial NO production by either Cav-1 deficiency or eNOS overexpression could disrupt the physiological balance between NO and EDH in microcirculation, resulting in impaired cardiovascular homeostasis in mice *in vivo*.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Altered Cardiovascular Phenotypes in Cav-1-KO and eNOS-Tg Mice

Systolic blood pressure was significantly lower in eNOS-Tg mice and tended to be lower in Cav-1-KO mice when

compared with wild-type (WT) mice (Table I in the online-only Data Supplement). Cav-1-KO mice exhibited concentric cardiac hypertrophy with 25% greater heart weight when compared with WT mice, although left ventricular systolic function per se was preserved. Plasma nitrite/nitrate levels in Cav-1-KO and eNOS-Tg mice were $\approx 2\times$ higher than those in WT mice (Table I in the online-only Data Supplement). These altered cardiovascular phenotypes are consistent with the previous studies.^{25,26}

Disrupted Balance Between NO and EDH in Endothelium-Dependent Relaxations in Cav-1-KO and eNOS-Tg Mice

Contraction responses to KCl (60 mmol/L) in the isolated aorta and mesenteric arteries were comparable among the 3 groups (Figure I in the online-only Data Supplement). Assessment of endothelium-dependent relaxations to acetylcholine of the aorta showed that NO was dominant in all genotypes in this conduit vessel, although Cav-1-KO mice showed enhanced NO-mediated relaxation responses and eNOS-Tg mice showed reduced maximal relaxation responses when compared with WT mice (Figure 1A–1C). In contrast, endothelium-dependent relaxations to acetylcholine of small mesenteric arteries from both Cav-1-KO and eNOS-Tg mice showed significantly enhanced NO-mediated relaxations as expected, whereas EDH-mediated responses were markedly reduced (Figure 1D–1F). The relative contributions of the 3 endothelium-derived relaxing factors to endothelium-dependent relaxations are summarized in Figure 1G for the aorta and Figure 1H for mesenteric arteries. In WT mice, endothelium-dependent relaxations were mostly mediated by NO in the aorta and by EDH in small mesenteric arteries, whereas in both Cav-1 KO and eNOS-Tg mice, this physiological balance was disrupted, resulting in the dominance of NO in both the aorta and the small mesenteric arteries (Figure 1G and 1H). Membrane potential recordings of small mesenteric arteries in the presence of indomethacin and N^ω-nitro-L-arginine (L-NNA) showed that Cav-1-KO mice exhibited attenuated EDH responses to acetylcholine (Figure 1I), whereas eNOS-Tg mice had less negative resting potentials when compared with WT mice (Figure 1J).

Endothelium-independent relaxations are shown in Figure II in the online-only Data Supplement. The VSMC responses to an NO donor sodium nitroprusside were markedly reduced in eNOS-Tg mice, whereas those of Cav-1-KO mice were slightly enhanced when compared with WT mice (Figure IIA and IIB in the online-only Data Supplement). In contrast, the relaxation responses to a K-channel opener NS-1619 were comparable among the 3 groups (Figure IIC and IID in the online-only Data Supplement). Figure IIE and IIF in the online-only Data Supplement shows VSMC responses to exogenous H₂O₂, a major EDH factor in mouse mesenteric arteries,³ in the presence of indomethacin and L-NNA. In mesenteric arteries (Figure IIF in the online-only Data Supplement), Cav-1-KO and eNOS-Tg mice showed slightly but significantly reduced relaxations to exogenous H₂O₂ when compared with WT mice (half-maximal effective concentration, EC₅₀ [μ mol/L]=17.4 \pm 1.2 in WT, 32.5 \pm 1.2 in Cav-1-KO,

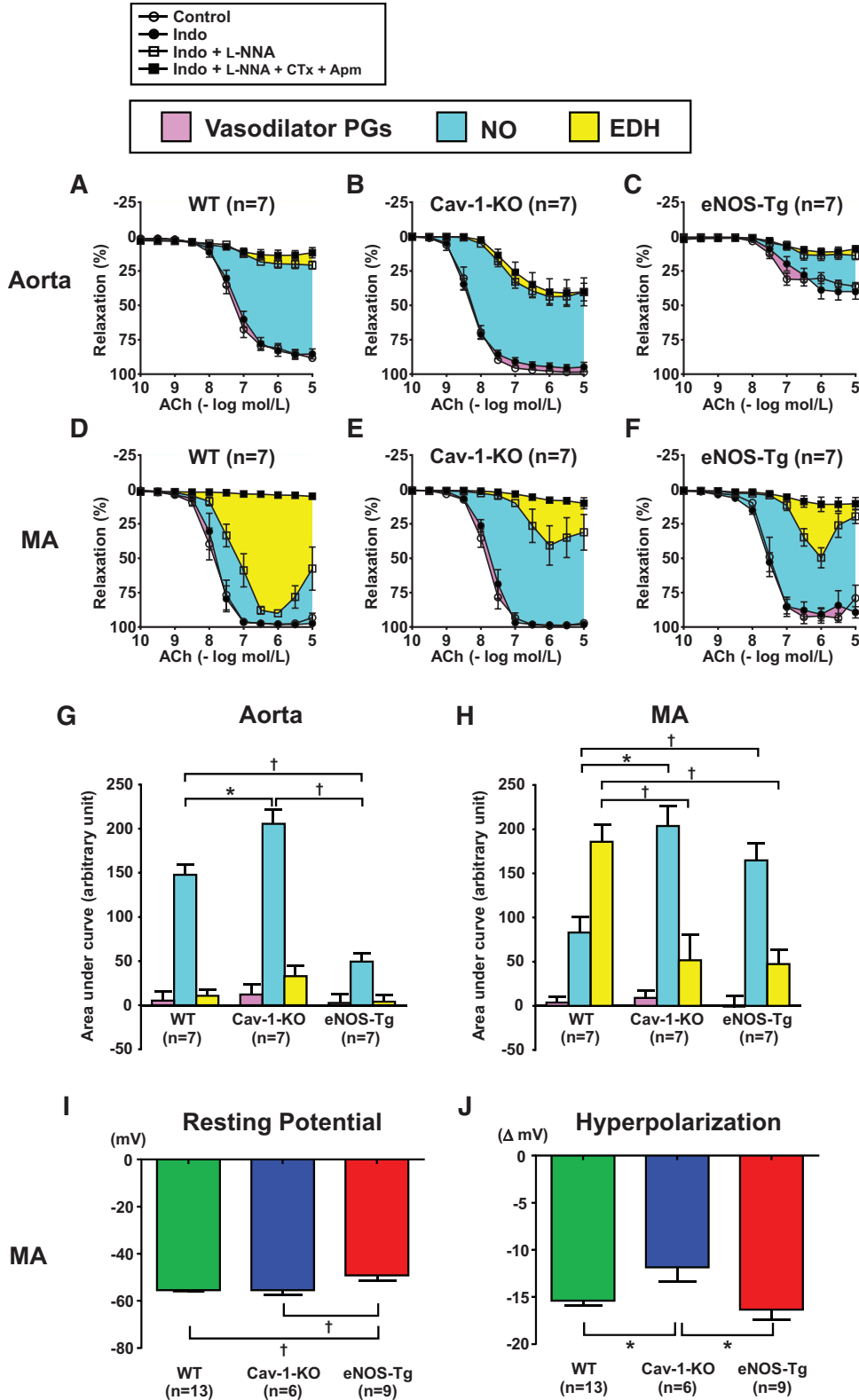


Figure 1. Endothelium-dependent relaxations and membrane potentials. **A–F**, Acetylcholine (ACh)-induced endothelium-dependent relaxations of aorta (**A**, **B**, and **C**) and mesenteric arteries (MAs; **D**, **E**, and **F**) from wild-type (WT; **A** and **D**), caveolin-1-knockout (Cav-1-KO; **B** and **E**), and endothelium-specific endothelial nitric oxide synthase transgenic (eNOS-Tg; **C** and **F**) mice are shown; n=7 animals per group. The contributions of vasodilator prostaglandins (vasodilator PGs; pink area), nitric oxide (NO; blue area), and endothelium-dependent hyperpolarization (EDH; yellow area) were determined by the inhibitory effect of indomethacin (Indo; 10^{-5} mol/L), N^o-nitro-L-arginine (L-NNA; 10^{-4} mol/L), and a combination of charybdotoxin (CTx; 10^{-7} mol/L) and apamin (Apm; 10^{-6} mol/L), respectively. **G** and **H**, The relative contributions of vasodilator PGs, NO, and EDH to endothelium-dependent relaxations among the 3 groups were compared with area under curve (**G**; aorta and **H**; MA). **I** and **J**, Membrane potentials of small mesenteric arteries in the presence of indomethacin (10^{-5} mol/L) and L-NNA (10^{-4} mol/L) were recorded using a glass capillary microelectrode; n=6 to 13 animals per group. **I**, Resting membrane potentials. **J**, Hyperpolarizations to ACh (10^{-5} mol/L). Values are expressed as mean \pm SEM. * P <0.05, † P <0.01.

and 29.1 ± 1.1 in eNOS-Tg mice). The precontraction forces to phenylephrine (10^{-6} mol/L) were comparable among the 3 groups in each condition (Table II in the online-only Data Supplement). Pretreatment of isolated vessels with a soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one increased the sensitivity to exogenous H_2O_2 -induced relaxations only in the vascular beds where NO-mediated responses were dominant (Figure IIIA–IIIF in the online-only Data Supplement).

Restoration of Impaired EDH-Mediated Relaxations by Reconstituting Cav-1 Back into Endothelium in Cav-1-KO Mice

Because Cav-1 is expressed almost ubiquitously in vascular wall including endothelial cells, VSMC, and adipocytes, we used a rescue model in which endothelial Cav-1 was reconstituted on the systemic Cav-1-deficient background (Figure IVA–IVC in the online-only Data Supplement). Notably, impaired EDH-mediated relaxations of small mesenteric arteries from

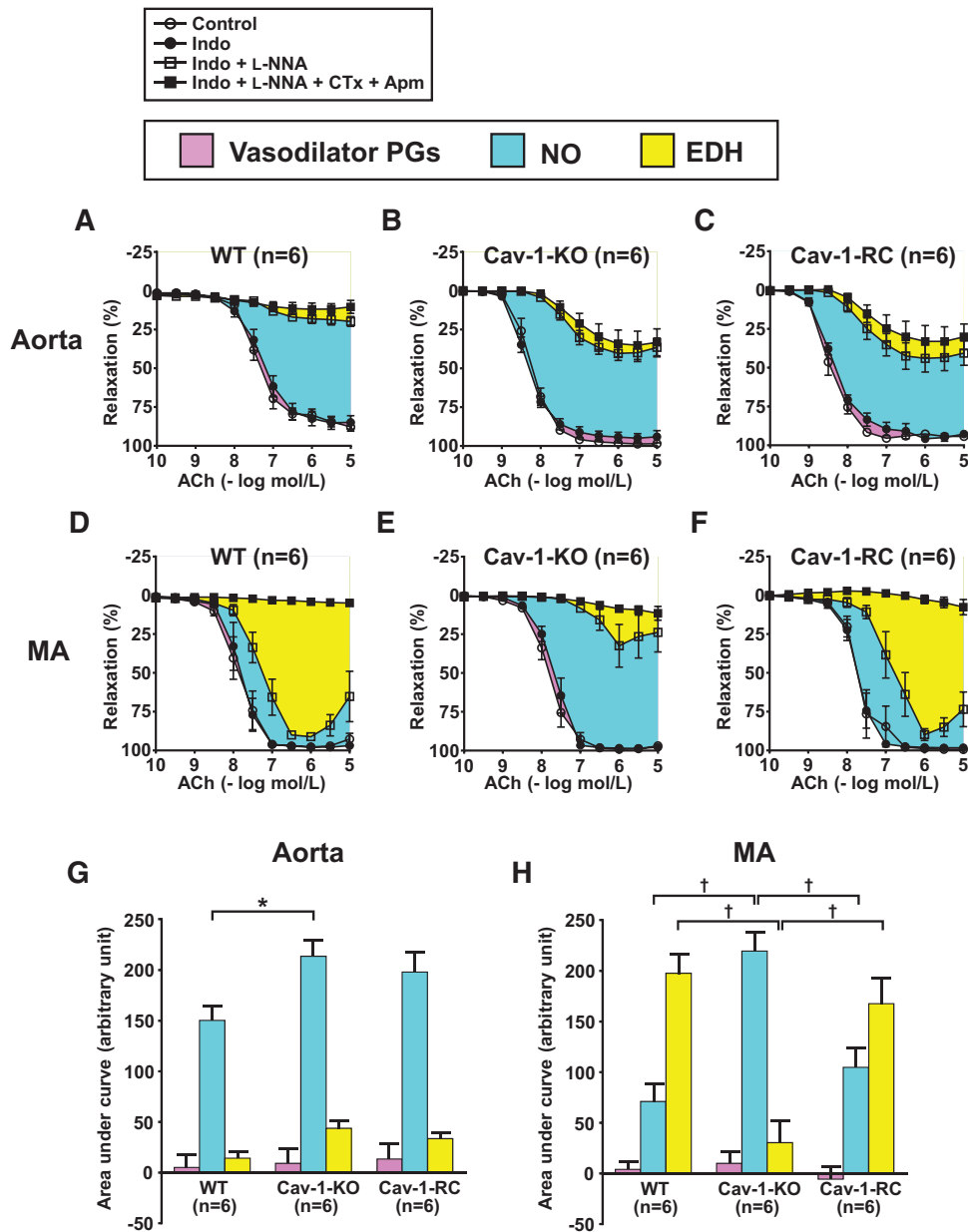


Figure 2. Endothelium-dependent relaxations. **A–F**, Acetylcholine (ACh)-induced endothelium-dependent relaxations of the aorta (**A**, **B**, and **C**) and mesenteric arteries (MAs; **D**, **E**, and **F**) from wild-type (WT; **A** and **D**), caveolin-1-knockout (Cav-1-KO; **B** and **E**), and endothelium-specific caveolin-1-reconstituted (Cav-1-RC; **C** and **F**) mice are shown; n=6 animals per group. The contributions of vasodilator prostaglandins (vasodilator PGs; pink area), nitric oxide (NO; blue area), and endothelium-dependent hyperpolarization (EDH; yellow area) were determined by the inhibitory effect of indomethacin (Indo; 10^{-5} mol/L), N^ω-nitro-L-arginine (L-NNA; 10^{-4} mol/L), and a combination of charybdotoxin (CTx; 10^{-7} mol/L) and apamin (Apm; 10^{-6} mol/L), respectively. **G** and **H**, The relative contributions of vasodilator PGs, NO, and EDH to endothelium-dependent relaxations among the 3 groups were compared with area under curve (**G**; aorta and **H**; MA). Values are expressed as mean±SEM. * $P < 0.05$, † $P < 0.01$.

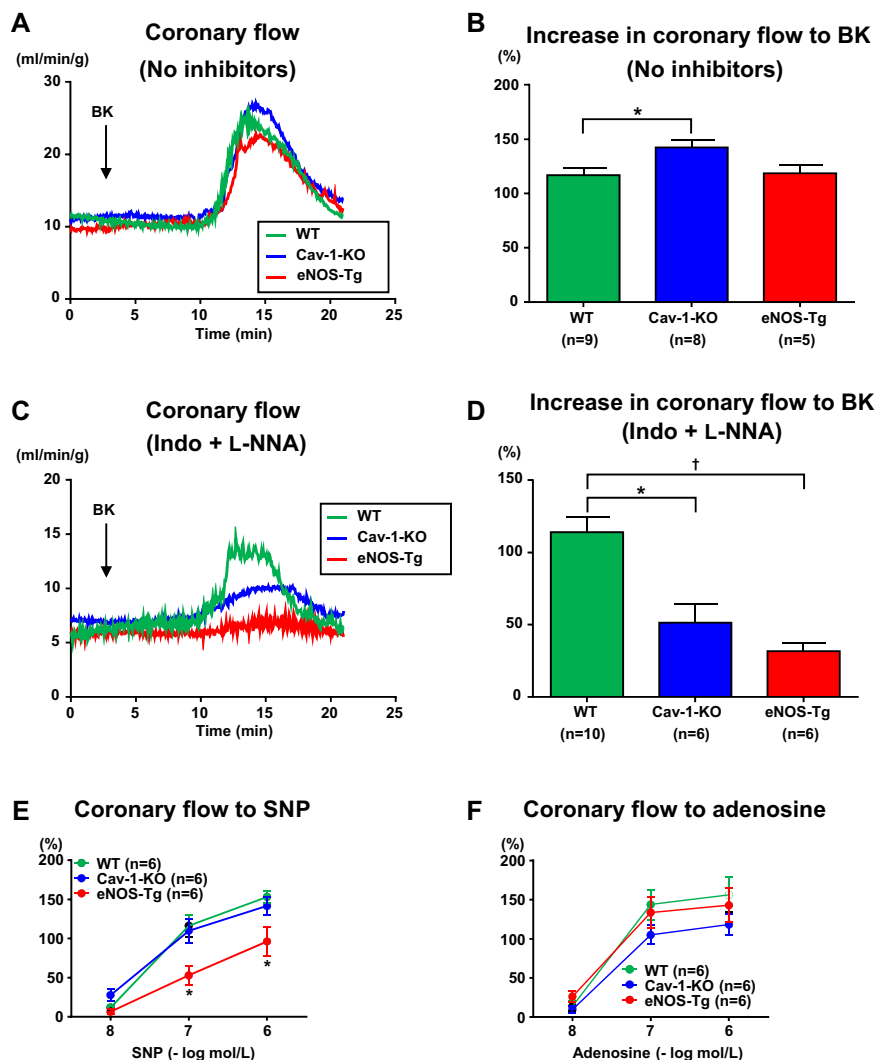


Figure 3. Coronary flow responses of Langendorff-perfused hearts. **A–D**, Representative traces and quantitative analysis of bradykinin (BK; 10^{-6} mol/L)-induced coronary flow changes in the absence (**A** and **B**) and presence (**C** and **D**) of indomethacin (Indo; 10^{-5} mol/L) and *N*^ω-nitro-L-arginine (L-NNA; 10^{-4} mol/L); $n=5$ to 10 animals per group. Coronary flows were normalized for heart weight and coronary flow changes are expressed as % change from the baseline flows. **E** and **F**, Coronary flow changes to sodium nitroprusside (SNP; **E**) and adenosine (**F**); $n=6$ animals per group. Values are expressed as mean \pm SEM. * $P < 0.05$, † $P < 0.01$ vs wild-type (WT). Cav-1-KO mice indicates caveolin-1-knockout mice; and eNOS-Tg mice, endothelium-specific endothelial nitric oxide synthase transgenic mice.

Cav-1-KO mice were completely rescued by crossing the mice with those with endothelium-specific overexpression of Cav-1 (Figure 2A–2H) with the VSMC responses unaffected (Figure VA–VF in the online-only Data Supplement). Although we obtained 3 lines of Cav-1 reconstituted mice with 2, 6, and 7 copies of the transgene, reduced EDH-mediated responses were restored in all the lines. These results demonstrate that reduced EDH-mediated relaxations in Cav-1-KO mice were attributable to the loss of Cav-1 in the endothelium.

Increased Basal NO Release in Cav-1-KO and eNOS-Tg Mice

To examine the effect of Cav-1 deficiency and eNOS overexpression on basal eNOS activity, intact vessels precontracted with a submaximal dose of phenylephrine were challenged with L-NNA as previously described.²⁷ The extent of basal NO release, when expressed as changes in basal tension in response to L-NNA, was comparable in the aorta among the 3 groups (Figure VIA and VIB in the online-only Data Supplement), but was significantly increased in mesenteric arteries of Cav-1-KO and eNOS-Tg mice (Figure VIC and VID in the online-only Data Supplement).

Reduced EDH-Mediated Coronary Flow Responses in Cav-1-KO and eNOS-Tg Mice

Next, we examined coronary microcirculation responses using the Langendorff-perfused heart model. Baseline coronary flows were comparable among the 3 groups in each condition, although indomethacin slightly increased and adding L-NNA on indomethacin reduced the basal flows (data not shown). Representative traces of coronary flow responses to bradykinin (10^{-6} mol/L) are shown in Figure 3A. In the absence of any inhibitors, bradykinin induced >2-fold increases in coronary flow in the 3 groups. Coronary flow responses to bradykinin were slightly but significantly higher in Cav-1-KO mice than in WT mice (Figure 3B). In contrast, the inhibitory effect of indomethacin on bradykinin-induced coronary flow increases was minimal (data not shown). Notably, in WT mice, bradykinin evoked substantial coronary flow increases that were resistant to a combination of indomethacin and L-NNA (Figure 3C and 3D), indicating that EDH-mediated responses play a primary role in the coronary microcirculation under normal condition. In contrast, EDH-mediated coronary flow responses to bradykinin were markedly reduced in both Cav-1-KO and eNOS-Tg mice (Figure 3C and 3D), consistent findings with isolated resistance vessels as described above. About endothelium-independent

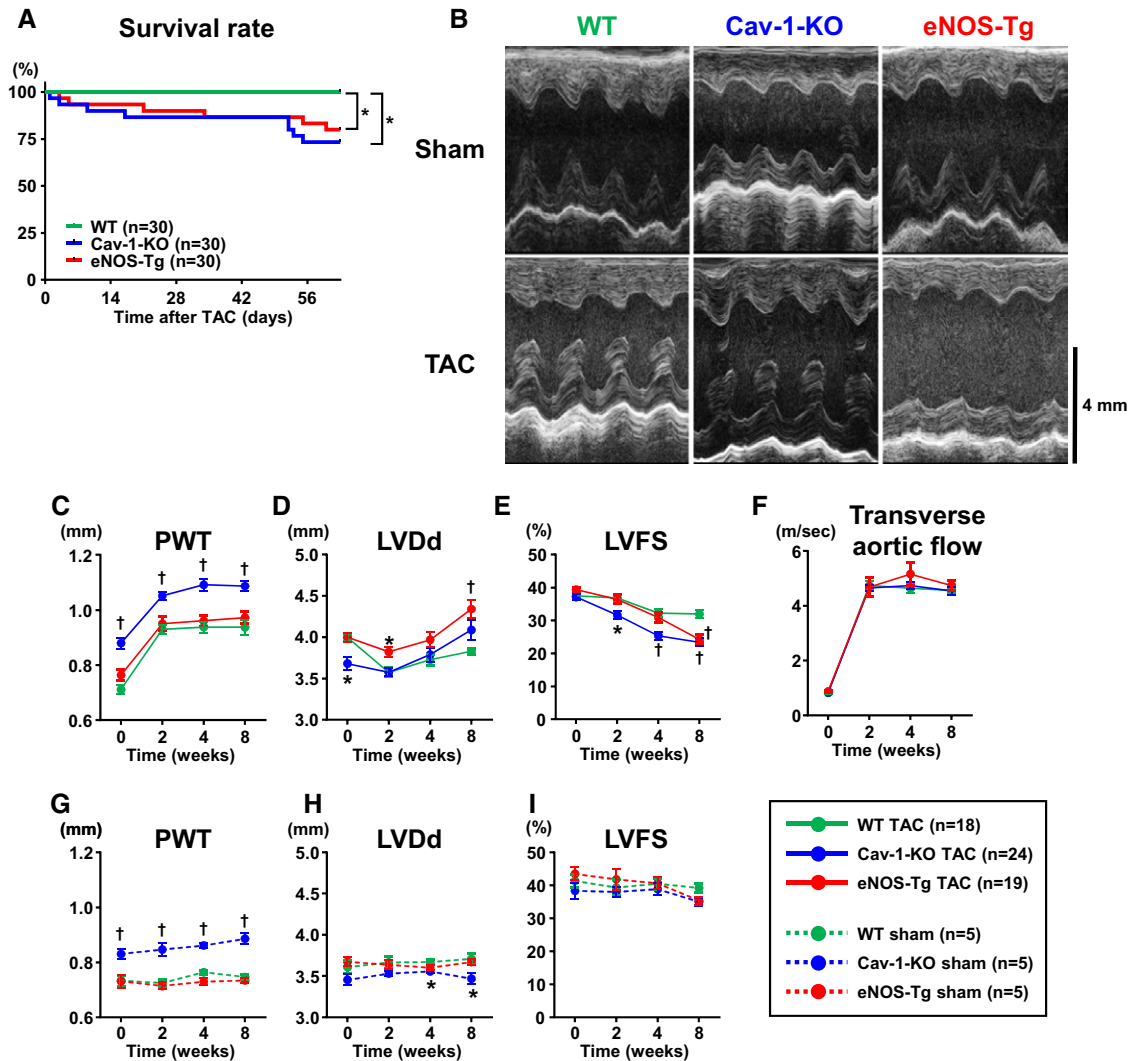


Figure 4. Effects of cardiac pressure overload on long-term survival and cardiac functions. **A**, Kaplan–Meier survival curves after transverse aortic constriction (TAC); n=30 animals per group. **P*<0.05 vs wild-type (WT; log-rank test). **B**, Representative images of echocardiography at 8 weeks after TAC or sham operations. **C–I**, Time course of echocardiographic parameters; n=18 to 24 animals per TAC group, n=5 animals per sham group. Values are expressed as mean±SEM. **P*<0.05, †*P*<0.01 vs WT at each time point. Cav-1-KO mice indicates caveolin-1-knockout mice; eNOS-Tg mice, endothelium-specific endothelial nitric oxide synthase transgenic mice; LVdD, left ventricular end-diastolic diameter; LVFS, LV fractional shortening; and PWT, posterior wall thickness.

VSMC responses, coronary flow increases to sodium nitropruside were significantly reduced in eNOS-Tg mice (Figure 3E), whereas those to adenosine were comparable among the 3 groups (Figure 3F), suggesting the selective desensitization of VSMC to NO in eNOS-Tg mice.

Impaired Cardiac Responses to Pressure Overload in Cav-1-KO and eNOS-Tg Mice in Vivo

To examine the impacts of the imbalance between NO and EDH in vivo, we imposed Cav-1-KO and eNOS-Tg mice to cardiac pressure overload by transverse aortic constriction (TAC). In this model, heart weights gradually increased with a peak at 4 weeks after TAC in all 3 groups (Figure VIIA in the online-only Data Supplement). TAC operation did not affect systolic blood pressures (Figure VIIB in the online-only Data Supplement) but slightly elevated heart rate from baseline to the same extent among the 3 groups (Figure VIIC in the online-only Data

Supplement). Importantly, both Cav-1-KO and eNOS-Tg mice showed significantly reduced survival rate during the 8-week follow-up period after TAC (Figure 4A), whereas no mice died in the sham groups. Serial echocardiography examinations showed that development of left ventricular systolic dysfunction was accelerated in Cav-1-KO and eNOS-Tg mice compared with WT mice (Figure 4B–4I), although comparable extent of pressure gradient across the aortic stenosis persisted among the 3 groups throughout the experimental period (Figure 4F). In contrast, in the sham groups, left ventricular systolic functions were comparable among the 3 groups, although Cav-1-KO mice exhibited concentric cardiac hypertrophy (Figure 4G–4I).

Impaired Coronary Flow Responses After TAC in Cav-1-KO and eNOS-Tg Mice

In the Langendorff experiments, bradykinin-evoked coronary flow increases were well maintained in WT mice even

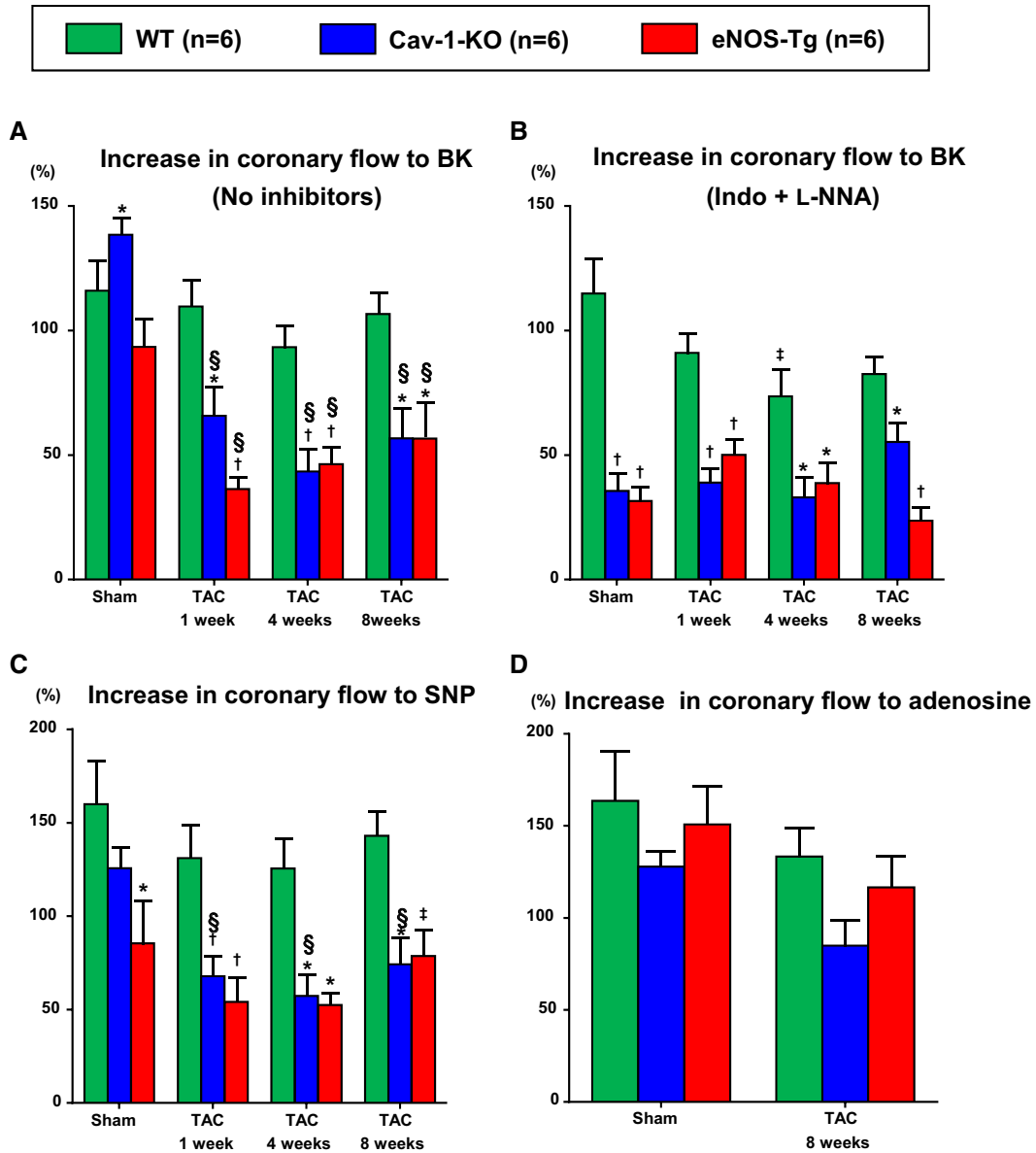


Figure 5. Effects of cardiac pressure overload on coronary flow responses. **A–D**, Time course of % coronary flow changes from baseline induced by bradykinin (BK; 10^{-6} mol/L) in the absence (**A**) and presence (**B**) of indomethacin (10^{-5} mol/L) and N^G -nitro-L-arginine (L-NNA; 10^{-4} mol/L), sodium nitroprusside (SNP; 10^{-6} mol/L; **C**), and adenosine (10^{-6} mol/L; **D**); $n=6$ animals per group. Values are expressed as mean \pm SEM. * $P<0.05$, † $P<0.01$ vs wild-type (WT) at each time point, ‡ $P<0.05$, § $P<0.01$ vs sham. Cav-1-KO mice indicates caveolin-1-knockout mice; and eNOS-Tg mice, endothelium-specific endothelial nitric oxide synthase transgenic mice.

after TAC (Figure 5A and 5B). In contrast, the responses were markedly reduced in Cav-1-KO and eNOS-Tg mice as early as at 1 week after TAC (Figure 5A), associated with reduced responses to sodium nitroprusside (Figure 5C). In Cav-1-KO and eNOS-Tg mice, EDH-mediated coronary flow responses (in the presence of indomethacin and L-NNA) were small before TAC and were fairly maintained throughout the study period after TAC (Figure 5B). However, coronary flow reserves assessed by the vasodilator responses to adenosine (10^{-6} mol/L) were relatively preserved in the 3 groups (Figure 5D). Taken together, these results indicate that NO-mediated coronary flow responses were more susceptible to cardiac pressure overload compared with EDH-mediated ones.

Accelerated Myocardial Relative Ischemia After TAC in Cav-1-KO and eNOS-Tg Mice

Finally, we examined histological changes of the heart after TAC operation. Among the 3 groups, cardiomyocyte hypertrophy (Figure VIIIA and VIIIB in the online-only Data Supplement) and perivascular (Figure VIIIC and VIID in the online-only Data Supplement) and interstitial (Figure VIIIE and VIIF in the online-only Data Supplement) fibrosis were equally developed after TAC. Similarly, myocardial capillary density, as evaluated by CD31 immunostaining, was comparable among the 3 groups after TAC (Figure 6A and 6B). Importantly, however, cardiac tissue hypoxia, known as relative ischemia in a hypertrophied heart,^{28,29} developed to a greater extent in Cav-1-KO and eNOS-Tg mice than in WT

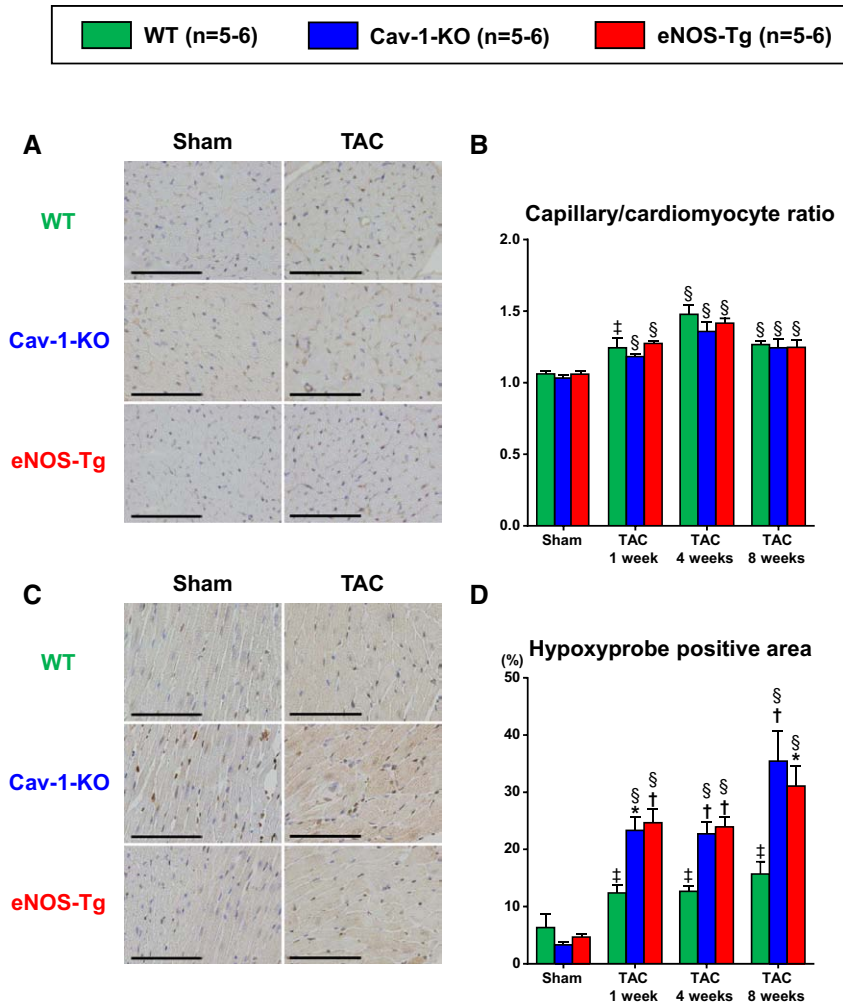


Figure 6. Histological analysis of capillary density and cardiac hypoxia. **A**, Representative immunostaining images of CD31-positive capillaries in the heart 8 weeks after transverse aortic constriction (TAC) or sham operations. **B**, Quantitative analysis of capillary density defined as the number of capillaries per cardiomyocyte. **C**, Representative immunostaining images of pimonidazole for detection of myocardial hypoxia 8 weeks after TAC or sham operations. **D**, Quantitative analysis of myocardial hypoxic area. Scale bars, 100 μ m; n=5 to 6 animals per group. Values are expressed as mean \pm SEM. * P <0.05, † P <0.01 vs WT at each time point, ‡ P <0.05, § P <0.01 vs sham. Cav-1-KO mice indicate caveolin-1-knockout mice; eNOS-Tg mice, endothelium-specific endothelial nitric oxide synthase transgenic mice; and WT, wild-type.

mice (Figure 6C and 6D), despite comparable extents of myocardial capillary density among the groups at each time point.

Discussion

The major findings of this study are that genetic disruption of the balance between NO and EDH toward NO dominance impaired EDH-mediated relaxations in isolated mesenteric arteries and perfused hearts ex vivo and accelerated left ventricular systolic dysfunction, reduced coronary flow reserve and enhanced myocardial hypoxia in response to pressure overload by TAC with reduced long-term survival in mice in vivo. To the best of our knowledge, this is the first study that demonstrates the importance of the physiological balance between NO and EDH to maintain cardiovascular homeostasis in vivo.

Interactions Between NO and EDH

In this study, to disrupt the balance between NO and EDH, we used Cav-1-KO²⁵ and eNOS-Tg mice.²⁶ As expected, NO-mediated relaxations were significantly enhanced, whereas EDH-mediated responses were markedly suppressed in mesenteric arteries in vitro and in perfused hearts ex vivo, resulting in the disruption of the physiological balance between NO and EDH (Figure IX in the online-only Data Supplement).

These altered endothelial functions are consistent with the previous findings obtained from the aorta of eNOS-Tg mice²⁶ and the aorta and mesenteric arteries of Cav-1-KO mice.^{23,25,30} Our findings are also in line with the previous reports that exogenous NO attenuates EDH-mediated responses in isolated rabbit carotid and porcine coronary arteries in vitro³¹ and canine coronary circulation in vivo.³² It was previously reported that NO exerts a negative feedback on endothelium-dependent relaxation through cyclic guanosine monophosphate-mediated desensitization in isolated canine coronary arteries³³ and that chronic treatment with nitroglycerin attenuates acetylcholine-induced hyperpolarization in rabbit aortic valve endothelial cells through increased oxidative stress.³⁴

Among the several candidates for the nature of EDH factor(s), we have previously demonstrated that endothelium-derived H₂O₂ plays a major role in EDH-mediated response in both mesenteric arteries¹³ and coronary circulation³⁵ in mice. Although the precise mechanisms by which NO suppresses EDH/H₂O₂ remain to be elucidated, desensitization of VSMC to EDH/H₂O₂ is likely to be involved as H₂O₂-induced protein kinase G1 α dimerization, a central mechanism of H₂O₂-induced vasodilatation,¹⁷ is inhibited by cyclic guanosine monophosphate-dependent activation of protein kinase G.³⁶ Indeed, in this study, resistance vessels from Cav-1-KO and eNOS-Tg mice

were approximately twice less sensitive to exogenous H_2O_2 when compared with WT mice. Moreover, soluble guanylate cyclase inhibition by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one sensitized mesenteric arteries from Cav-1-KO and eNOS-Tg mice, but not those from WT mice, to H_2O_2 -induced vasodilatation. Taken together, these results support the idea that excessive endothelium-derived NO desensitized blood vessels to EDH/ H_2O_2 -mediated relaxations. Another possible explanation for the reduced EDH-mediated responses is reduced production of EDH/ H_2O_2 . This notion is based on our previous finding that the extent of eNOS bound to Cav-1 is greater in mesenteric arteries than in the aorta and eNOS is functionally inhibited in resistance vessels through Cav-1-dependent mechanism, switching its function from NO synthase to EDH/ H_2O_2 -generating enzyme in mice under physiological condition.²³ It is conceivable that both Cav-1 deficiency and eNOS overexpression may lead to disequilibrium between eNOS and Cav-1 in resistance vessels with a resultant shift from EDH/ H_2O_2 to NO generation by eNOS in microcirculations.

In addition, the actions of other EDH factors are also reported to be inhibited by NO. For example, the earlier report by Bauersachs et al³¹ showed that exogenous NO donors could attenuate EDH-mediated relaxations *in vitro*. A putative mechanism involved in this phenomenon is the inhibitory effect of NO on epoxyeicosatrienoic acid production through inhibition of cytochrome P450 epoxygenase activity.³¹ It is important to examine whether the findings by Bauersachs et al³¹ in isolated rabbit carotid arteries can be extrapolated to other blood vessels, including resistance vessels *in vivo*. More recently, Mustafa et al³⁷ have reported that NO exerts a direct inhibitory effect on cystathionine γ -lyase activity *in vitro*. Given that cystathionine γ -lyase is a biosynthetic enzyme of hydrogen sulfide that has been shown to be one of EDH factors in mouse mesenteric arteries,^{12,37} it is possible that this mechanism is also involved in the negative feedback of NO on EDH-mediated relaxations. However, it remains to be examined whether hydrogen sulfide is an EDH factor in coronary arteries.

Impaired Cardiovascular Homeostasis in Cav-1-KO and eNOS-Tg Mice

Endogenous NO has been shown to exert protective roles against cardiovascular diseases through multiple mechanisms, including vasodilatation (mainly in large conduit vessels), inhibition of platelet aggregation, and prevention of thrombosis.⁴ Despite the enhanced NO-mediated responses, both Cav-1-KO and eNOS-Tg mice showed reduced survival in response to cardiac pressure overload *in vivo*, associated with accelerated cardiac systolic dysfunction and myocardial ischemia in the hypertrophied hearts. About the explanation for higher heart weight in Cav-1-KO mice at baseline, it has been reported that Cav-1-KO mice develop spontaneous cardiac hypertrophy through hyperactivation of p42/44 MAP kinase and Akt pathways.^{38,39} This raises the possibility that the baseline differences in heart weight could affect the responses to cardiac pressure overload. However, comparable extent of pressure gradient across the aortic stenosis persisted (Figure 4F), and the relative increase in heart weight/body weight after TAC was comparable among the 3 groups (Figure VIIA in the online-only Data Supplement). In addition, baseline coronary flow per gram heart weight was also

comparable among the 3 groups (WT, 11.8 ± 0.8 ; Cav-1-KO, 11.5 ± 0.8 ; eNOS-Tg, and 12.2 ± 1.4 mL/min per gram). Under these conditions, both Cav-1-KO and eNOS-Tg mice showed that coronary flow increases to bradykinin, which were mainly mediated by NO, were markedly reduced after TAC in a similar manner (Figure 5A and 5B).

Several pathogenic mechanisms of coronary microvascular dysfunction have been proposed, including structural (eg, vascular remodeling, vascular rarefaction, perivascular fibrosis, etc.) and functional alterations (eg, endothelial dysfunction, dysfunction of VSMC, etc.).⁴⁰ Using TAC model, several previous studies showed that insufficient angiogenesis, which is often regarded as a main cause of cardiac hypertrophy in maladaptive responses, occurred in a hypertrophied heart, leading to myocardial ischemia and resultant heart failure.^{28,29} However, in this study, severe cardiac tissue hypoxia in Cav-1-KO and eNOS-Tg mice after TAC operation cannot be explained from such a morphological view point because the extents of perivascular fibrosis and capillary density in response to cardiac pressure overload (TAC) were comparable among the 3 groups. Because NO-mediated coronary flow responses were more susceptible to cardiac pressure overload, as noted in Cav-1-KO and eNOS-Tg mice, the shift from EDH to NO in resistance vessels seems to be associated with worse outcomes after TAC operation; the reduced EDH-mediated responses in resistance vessels lead to insufficient tissue perfusion as evidenced by the exacerbated cardiac hypoxia in Cav-1-KO and eNOS-Tg mice after TAC. In addition, endothelium-dependent and endothelium-independent coronary flow responses were relatively preserved in WT mice after TAC, which are consistent with the previous studies with isolated guinea pig heart⁴¹ and isolated porcine subendocardial arterioles.⁴² However, in Cav-1-KO and eNOS-Tg mice, the coronary flow responses to sodium nitroprusside were significantly reduced, whereas those to adenosine were preserved, suggesting that NO tolerance was developed after TAC in those mice.⁴³ These results are consistent with the widely accepted notion that EDH works as a compensatory vasodilator system when NO-mediated relaxations are hampered. Thus, EDH dominance in microcirculation is a vital mechanism to maintain sufficient tissue perfusion in the setting of cardiac pressure overload where NO-mediated responses are compromised.

Study Limitations

Several limitations should be mentioned for this study. First, the precise molecular mechanisms by which excessive NO disrupted EDH-mediated vasodilatation remain to be elucidated. Second, although both Cav-1-KO and eNOS-Tg mice showed reduced EDH-mediated responses associated with enhanced NO-mediated responses in microcirculations, some phenotypes were different between the 2 genotypes; spontaneous cardiac hypertrophy was noted only in Cav-1-KO mice and systemic hypotension only in eNOS-Tg mice. One possible explanation for this discrepancy is that systemic Cav-1-KO mice were used in this study. Although Cav-1 is expressed not only in endothelial cells but also in various types of cells in vascular wall,⁴⁴ reexpression of Cav-1 in the endothelium was enough to restore the impaired EDH-mediated relaxations, suggesting the primarily role of endothelial Cav-1 in EDH-mediated responses. A genetic ablation of Cav-1 in a cell-specific manner

(eg, endothelium-specific Cav-1-KO model) may enable us to further clarify this point. Third, cardiac work and contractility were not determined in the Langendorff experiments. Although the coronary flows were measured under a constant pressure at a constant pacing rate and the baseline coronary flows were comparable among the groups in each condition, a possible difference in cardiac work among the 3 groups cannot be fully ruled out. Fourth, although we have previously demonstrated that H₂O₂ is a major EDH factor in human mesenteric arteries⁴⁵ as well as in mouse mesenteric arteries,^{3,5,13} it remains to be examined whether the present findings obtained with the mice can be extrapolated to humans. All these important issues remain to be examined in future studies.

Clinical Implications

Accumulating evidence suggests that NO and EDH share the roles in modulating vasodilatation in a vessel size-dependent manner; NO plays a dominant role in relatively large arteries and EDH in resistance vessels. In various pathological conditions with atherosclerotic risk factors, NO-mediated relaxations are easily impaired, whereas EDH-mediated responses are fairly preserved or even enhanced to maintain vascular homeostasis.^{3,46} Our present findings shed new light on the significance of maintaining EDH-mediated relaxations in microcirculation to develop a novel therapeutic strategy; upregulation of eNOS is not always effective as it could cause imbalance between NO and EDH. Although nitrate therapies have been shown to be effective in many clinical trials,^{47,48} these beneficial effects do not necessarily come from immediate actions of NO (ie, in combination with hydralazine⁴⁷ and nitrate-rich beet fruit juice⁴⁸). In contrast, several previous large-scale clinical studies have shown that chronic nitrate therapies for patients with myocardial infarction did not improve mortality rate⁴⁹ or even worsened their prognosis,²⁴ and their routine long-term use is not recommended in the current guidelines.^{50,51} The notion that excessive NO disrupts EDH-mediated responses may provide insight into the potential harmful effects of chronic nitrate therapy.

Conclusions

In conclusion, we were able to demonstrate that the physiological balance between NO and EDH plays a crucial role in maintaining cardiovascular homeostasis in mice in vivo.

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Disclosures

None.

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Significance

Endothelium-derived nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) share the roles in modulating vasodilatation in a vessel size-dependent manner; NO plays a dominant role in conduit arteries and EDH in resistance vessels, however, the importance of the balance between NO and EDH in cardiovascular homeostasis remains to be elucidated. The major findings of this study were that genetic disruption of the balance between NO and EDH toward NO dominance in mice causes reduced EDH-mediated relaxations in microcirculations, and the imbalance between NO and EDH leads to accelerated left ventricular systolic dysfunction, reduced coronary flow reserve and enhanced myocardial hypoxia in response to chronic pressure overload by transverse aortic constriction with reduced long-term survival in mice in vivo. These findings shed new light on the significance of maintaining EDH-mediated relaxations in microcirculation to develop a novel therapeutic strategy for cardiovascular diseases, providing a clue for better understanding of potential harmfulness of chronic nitrate therapy.