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. In 1988, Feletou and Vanhoutte and Chen et al 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, electric communication through gap junctions, K+ ions, hydrogen sulfide, and as we have originally identified and other researchers have subsequently confirmed, endothelium-derived hydrogen peroxide (H2O2). Intriguingly, the contribution of endothelium-derived relaxing factors to endothelium-dependent vasodilation 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. 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 pressure and vascular metabolic functions in general and coronary autoregulation and metabolic dilatation in particular.

We have previously demonstrated the diverse roles of the NO synthases (NOSs) system in the endothelium depending...
on blood vessel size. 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 $\text{H}_2\text{O}_2$-induced protein kinase G1$\alpha$ dimerization and subsequent activation of potassium channels, leading to hyperpolarization and vasodilatation.

Furthermore, we have recently demonstrated that endothelial nitric oxide synthase (eNOS) serves as a NO-generating system to cause vasodilatation via soluble guanylate cyclase–cyclic guanosine monophosphate. However, the importance of the physiological balance between NO and EDH 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, suggesting 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 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 compared with wild-type (WT) mice. Compared with WT mice, 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 ≈2× higher than those in WT mice. These altered cardiovascular phenotypes are consistent with the previous studies.

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 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 $\text{N}^\text{ω}$-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 $\text{H}_2\text{O}_2$, a major EDH factor in mouse mesenteric arteries, 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 $\text{H}_2\text{O}_2$ when compared with WT mice (half-maximal effective concentration, EC$_{50}$ [μmol/L]=17.4±1.2 in WT, 32.5±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\textomega-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±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 H₂O₂-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

![Graphs showing endothelium-dependent relaxations](image-url)
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.7 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 -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
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VSMC responses, coronary flow increases to sodium nitroprusside 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
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; D) 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 VIII A and VIIIB in the online-only Data Supplement) and perivascular (Figure VIIIC and VIIID in the online-only Data Supplement) and interstitial (Figure VIIIE and VIIIF 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, was developed to a greater extent in Cav-1-KO and eNOS-Tg mice than in WT.
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-KO and eNOS-Tg mice. 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 mice and the aorta and mesenteric arteries of Cav-1-KO mice. 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 vitro and canine coronary circulation in vivo. It was previously reported that NO exerts a negative feedback on endothelium-dependent relaxation through cyclic guanosine monophosphate-mediated desensitization in isolated canine coronary arteries and that chronic treatment with nitroglycerin attenuates acetylcholine-induced hyperpolarization in rabbit aortic valve endothelial cells through increased oxidative stress.

Among the several candidates for the nature of EDH factor(s), we have previously demonstrated that endothelium-derived H$_2$O$_2$ plays a major role in EDH-mediated response in both mesenteric arteries and coronary circulation in mice. Although the precise mechanisms by which NO suppresses EDH/H$_2$O$_2$ remain to be elucidated, desensitization of VSMC to EDH/H$_2$O$_2$ is likely to be involved as H$_2$O$_2$-induced protein kinase G1α dimerization, a central mechanism of H$_2$O$_2$-induced vasodilatation, is inhibited by cyclic guanosine monophosphate-dependent activation of protein kinase G. Indeed, in this study, resistance vessels from Cav-1-KO and eNOS-Tg mice...
were approximately twice less sensitive to exogenous H2O2 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 H2O2-induced vasodilatation. Taken together, these results support the idea that excessive endothelin-derived NO desensitized blood vessels to EDH/H2O2-mediated relaxations. Another possible explanation for the reduced EDH-mediated responses is reduced production of EDH/H2O2. 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/H2O2-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/H2O2 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 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 al32 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 (e.g., vascular remodeling, vascular rarefaction, perivascular fibrosis, etc.) and functional alterations (e.g., endothelial dysfunction, dysfunction of VSMC, etc.).41 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 heart40 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 these 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 arteries as well as in mouse mesenteric arteries, 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. 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; these beneficial effects do not necessarily come from immediate actions of NO (ie, in combination with hydralazine and nitrate-rich beet fruit juice). In contrast, several previous large-scale clinical studies have shown that chronic nitrate therapies for patients with myocardial infarction did not improve mortality rate or even worsened their prognosis, and their routine long-term use is not recommended in the current guidelines. 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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Physiological Balance Between NO and EDH

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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.
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Detailed Methods

Animals
This study was reviewed and approved by the Committee on Ethics of Animal Experiments of Tohoku University (2013MdA-492, 2014MdA-021, 2014MdA-206). Male C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan), caveolin-1-knockout (Cav-1-KO) mice\textsuperscript{1} from Jackson Laboratory (Bar Harbor, ME) and endothelium-specific endothelial nitric oxide synthase transgenic (eNOS-Tg) mice\textsuperscript{2} were a generous gift from Drs. Emoto (Kobe Pharmaceutical University, Japan) and Hirata (Kobe University, Japan). Both Cav-1-KO and eNOS-Tg mice were backcrossed to C57BL/6 mice more than 10 generations and thus C57BL/6 mice served as a wild-type (WT) control. We generated endothelium-specific Cav-1-transgenic mice as described previously.\textsuperscript{2,3} Briefly, canine Cav-1 cDNA (537bp) was subcloned into the NotI site of the plasmid vector consisting of murine Tie2 promotor-SV40 intron polyA-murine Tie2 enhancer, linearized by NotI-SalI digestion and microinjected into fertilized eggs prepared from C57BL/6 mice. Endothelium-specific Cav-1-reconstituted (Cav-1-RC) mice were generated by crossing endothelium-specific Cav-1 transgenic mice with Cav-1-KO mice (Figure S4A). All animals were cared for in accordance with the rules and regulations configured by the committee, fed standard rodent chow and maintained on 12-h light/dark cycles.\textsuperscript{4} Systolic blood pressure and heart rate were measured in conscious mice using a tail-cuff method (BP-2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC).\textsuperscript{5}

Plasma Nitrite/Nitrate Levels
Mice fasted for 15 hours were euthanized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and blood samples were collected from the heart. After centrifugation at 7,000 rpm for 15 min at 4 °C in the presence of ethylenediaminetetraacetate (EDTA; 0.5 mmol/L), the plasma was collected and nitrite/nitrate levels were measured by the Griess Reagent Kit (Dojindo Laboratories, Kumamoto, Japan).\textsuperscript{6}
Organ Chamber Experiments

We measured isometric tensions of the aorta and the first branch of mesenteric arteries (approximately 200-250 μm in outer diameter) as described previously. After mice were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), the aorta and mesenteric arteries were carefully isolated under a microscope, cut into 1-mm length rings without adventitia and mounted in a wire myograph (620M, Danish Myo Technology, Aarhus, Denmark). The arterial rings were bathed in 5 mL Krebs-Henseleit buffer (KHB) aerated with 95% O₂ plus 5% CO₂ at 37 °C, stretched to optimal resting tensions. After 60-min equilibration period, the rings were challenged with KCl (60 mmol/L) to confirm their viability; rings that were able to generate over 1 mN of force were allowed for the following isometric tension recordings.

After washout and 30-min recovery period, the rings were precontracted with phenylephrine (10⁻⁶ mol/L) to examine the relaxation responses to acetylcholine (ACh, 10⁻¹⁰ to 10⁻⁵ mol/L). The relaxation responses were calculated as a percentage of the precontraction forces induced by phenylephrine. The contributions of vasodilator prostaglandins, nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) to ACh-induced endothelium-dependent relaxations were determined by the inhibitory effect of indomethacin (10⁻⁵ mol/L), N⁶-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. All the inhibitors were applied to organ chambers 30 min before precontraction with phenylephrine. To assess endothelium-independent relaxations, a NO donor sodium nitroprusside (SNP, 10⁻¹⁰ to 10⁻⁵ mol/L) and a K channel opener NS-1619 (10⁻⁸ to 10⁻⁴ mol/L) were used. Vascular responses to exogenous hydrogen peroxide (H₂O₂) (10⁻⁸ to 10⁻³ mol/L) were examined in the presence of indomethacin (10⁻⁵ mol/L) and L-NNA (10⁻⁴ mol/L) or in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 20 μmol/L). To measure basal NOS activity, vessels were precontracted with a submaximal dose of phenylephrine (10⁻⁷ mol/L) and at the plateau of phenylephrine-induced contraction, L-NNA (10⁻⁴ mol/L) was challenged to inhibit basally-released NO. The responses were
Electrophysiological Experiments

Membrane potentials of mesenteric arteries were recorded as described previously.4 Briefly, the rings of small mesenteric arteries were immersed in an organ chamber filled with KHB gassed with 95% O₂ plus 5% CO₂ at 37°C. A glass capillary microelectrode was impaled into vascular smooth muscle cells from the adventitial side. Resting potentials and changes in the membrane potentials evoked by ACh were recorded in the presence of indomethacin (10⁻⁵ mol/L) and L-NNA (10⁻⁴ mol/L). The responses were monitored on a computer-based analysis system in LabChart 7.0 software.⁴

Langendorff Experiments

Langendorff experiments were performed as described previously.⁴⁹¹⁰ Mice were premedicated intraperitoneally with heparin (1,000 units), then 10 min later they were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The heart was cut off into iced KHB containing 0.5 mmol/L EDTA to be arrested. After trimming of the excess extra cardiac tissues, the ascending aorta was canulated with a blunted 21-gauge needle under a microscope and tied with 2 sutures. Then, the canulated heart was mounted onto a Langendorff apparatus (Model IPH-W2, Primetech Corporation, Tokyo, Japan) to be perfused retrogradely with 37°C KHB aerated with 95% O₂ plus 5% CO₂, returning spontaneously to vigorous beating. The preparation above was performed as quickly as possible to minimize cardiac damage. An air bubble trap (Physio-Tech, Tokyo, Japan) and a sterile filter with 0.45-μm pore (Merck Millipore, Darmstadt, Germany) were equipped just above the heart to avoid air embolization of the coronary arteries. After a 10-min stabilization period, the heart was paced at 400 bpm and perfused at a constant pressure of 80 mmHg. Coronary flow changes to agonists (bradykinin, adenosine, and SNP) were measured with a flowmeter (FLSC-01, Primetech Corporation, Tokyo, Japan) placed in the Langendorff circuit and monitored by a computer-based analysis system in
EDH-mediated responses were determined in the presence of indomethacin ($10^{-5}$ mol/L) and L-NNA ($10^{-4}$ mol/L).

**Transverse Aortic Constriction**

Transverse aortic constriction (TAC) was performed with slight modification. Briefly, mice at 8-12 weeks of age weighing 25±2 g were anesthetized with isoflurane (1.5-2.0%), intubated and ventilated with a mouse ventilator (MiniVent Type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). Between the right innominate and left carotid arteries, the transverse aorta was constricted with a 7-0 silk suture against a 25-gauge blunted needle, which was removed immediately after the ligation to yield a constriction of 0.51 mm in diameter. Sham-operated mice were subjected to the same procedure without TAC. After the operations, the mice were followed up for 8 weeks to evaluate the long-term survival rate, cardiac function, and coronary flow responses.

**Echocardiography**

We performed transthoracic echocardiography using the Vevo 2100 Imaging System (VisualSonics, Toronto, Canada) at baseline and 2, 4 and 8 weeks after the TAC operation. Mice were secured on a warm stage at 37°C and lightly anesthetized with 0.5-0.8% isoflurane to maintain the heart rate at approximately 500 bpm. Left ventricular (LV) end-diastolic diameter, LV end-systolic diameter, intraventricular septal thickness, posterior wall thickness and LV fractional shortening were obtained from M-mode short-axis images at the papillary muscle level. To evaluate the extent of pressure gradient across the aortic banding site, the transverse aortic flow velocity was measured with pulse wave Doppler. All measurements were averaged from 3 to 5 beats.

**Histological Analysis**

After perfusion fixation with 10% buffered formalin was performed via the inferior vena cava, the hearts, aorta and mesenteries were harvested, immersed in 10% buffered formalin for 24 h,
embedded in paraffin and cut into 3-μm-thick sections. The serial sections were stained with hematoxylin-eosin for evaluation of the cross-sectional area of cardiomyocytes, with Masson-trichrome for that of interstitial or perivascular fibrosis area, and CD31 (PECAM-1, 1:400 dilution; BD Pharmingen, #550274) for that of capillary density and Cav-1 (1:1,600 dilution; Cell Signaling Technology, #3267). Cross-sectional area, fibrosis area and capillary density were calculated as described previously. To assess the cardiac tissue hypoxia, pimonidazole (HypoxyprobeTM-1 Omni Kit, Hypoxyprobe, Burlington, MA) was used according to the manufacturer’s instructions. This method is frequently employed to evaluate hypoxia in various tissues including mouse hearts. Pimonidazole was injected intaperitoneally at a dosage of 60 mg/kg body weight 30 min prior to a sample harvest. Following the injection, pimonidazole distributes to all tissues to form chemical adducts with thiol containing proteins only in those cells that have a low oxygen concentration (pO2 less than 10 mmHg). The chemical adducts were immunostained with an affinity purified rabbit anti-pimonidazole antibody. Slides were viewed with a light microscope (BX51, Olympus, Tokyo, Japan) and analyzed by DP Controller, DP Manager software (Olympus, Tokyo, Japan) and Image J Software (NIH, Bethesda, MD).

**Drugs and Solution**

CTx was purchased from Peptide Institute (Osaka, Japan), SNP from Maruishi Seiyaku (Osaka, Japan) and all other drugs from Sigma Aldrich (St. Louis, MO). Indomethacin was dissolved in 10 mmol/L Na2CO3, NS-1619 and ODQ in dimethyl sulfoxide, and the others in distillated water. The ionic composition of KHB was as follows (mmol/L): Na+ 144, K+ 5.9, Mg2+ 1.2, Ca2+ 2.5, H2PO4− 1.2, HCO3− 24, Cl− 129.7, and glucose 5.5.

**Statistical Analysis**

Values are expressed as means±SEM. All parameters were evaluated with the two tailed unpaired Student’s t-test or compared by one-way analysis of variance (ANOVA), followed by
Tukey’s test for multiple comparisons. Dose response curves between groups were compared by two-way ANOVA, followed by Tukey’s multiple comparisons tests. Survival curves were plotted according to the Kaplan-Meier method and evaluated with log-rank test. Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA). Values of $P<0.05$ were considered to be statistically significant.
Supplemental References

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Supplemental Figure I. KCl-induced contractions. 

A and B, KCl (60 mmol/L)-induced contractions of the aorta (A) and those of mesenteric arteries (MA) (B). n = 7 animals per group. Results are expressed as means ± SEM.
Supplemental Figure II. Endothelium-independent relaxations.

A-F. Endothelium-independent relaxations of aorta (A, C and E) and mesenteric arteries (MA) (B, D and F) to sodium nitroprusside (SNP) (A and B), NS-1619 (C and D) and hydrogen peroxide (H$_2$O$_2$) (E and F) are shown. n = 6-7 animals per group. The responses to H$_2$O$_2$ were examined in the presence of indomethacin (10$^{-5}$ mol/L) and N$^\omega$-nitro-L-arginine (L-NNA; 10$^{-4}$ mol/L). Values are expressed as means±SEM. *P<0.05, †P<0.01 vs. WT.
Supplemental Figure III. Effects of sGC inhibition on \( \text{H}_2\text{O}_2 \)-induced relaxations. 

A-F, The responses of the aorta (A, C and E) and mesenteric arteries (MA) (B, D and F) to exogenous hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) were examined in the presence or absence of a soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 20 \( \mu \text{mol/L} \)). \( n = 7 \) animals per group. Values are expressed as means\( \pm \)SEM. *\( P<0.05 \), †\( P<0.01 \) vs. control.
Supplemental Figure IV. Endothelium-specific Cav-1 reconstitution on Cav-1-KO background.

A, The construct used to generate endothelium-specific Cav-1 transgenic mice. The canine Cav-1 cDNA (537 bp) was driven under the endothelium-specific promotor, murine Tie2 promotor. Endothelium-specific Cav-1-reconstituted (Cav-1-RC) mice were generated by crossing endothelium-specific Cav-1 transgenic mice with Cav-1-KO mice. B and C, Immunostaining images for Cav-1 and CD31 (an endothelial cell marker) of the aorta (B) and mesenteric arteries (C). Scale bars = 50 μm.
Supplemental Figure V. Endothelium-independent relaxations.
A-F, Endothelium-independent relaxations of the aorta (A, C and E) and mesenteric arteries (MA) (B, D and F) to sodium nitroprusside (SNP) (A and B), NS-1619 (C and D) and hydrogen peroxide (H₂O₂) (E and F) are shown. n = 6 animals per group. The responses to H₂O₂ were examined in the presence of indomethacin (10⁻⁵ mol/L) and Nω-nitro-L-arginine (L-NNA; 10⁻⁴ mol/L). Values are expressed as means±SEM.
*P<0.05, †P<0.01 vs. WT.
Supplemental Figure VI. Basal NO release.
A-D, Representative traces and quantitative analysis of contractile responses evoked by L-NNA (10^-4 mol/L) in rings precontracted with a submaximal dose of phenylephrine (PE; 10^-7 mol/L) from aorta (A and B) and mesenteric arteries (C and D). n = 7 animals per group. Values are expressed as means±SEM. *P<0.05, †P<0.01.
Supplemental Figure VII. Effects of cardiac pressure-overload on survival and hemodynamic parameters.

A–C, Time course of heart weight/body weight changes (n = 6 per group) (A), systolic blood pressure (B) and heart rate (C) (n = 11–18 animals per TAC-group, n = 5 animals per sham-group) after transverse aortic constriction (TAC) or sham operations. Values are expressed as means ± SEM. *P<0.05, †P<0.01 vs. WT at each time point, ‡P<0.05, §P<0.01 vs. sham.
Supplemental Figure VIII

A

WT

Cav-1-KO

eNOS-Tg

B

Myocyte cross-sectional area

(\mu m^2)

Sham TAC 1 week TAC 4 weeks TAC 8 weeks

C

WT

Cav-1-KO

eNOS-Tg

D

Perivascular fibrosis

(\%)

Sham TAC 1 week TAC 4 weeks TAC 8 weeks

E

WT

Cav-1-KO

eNOS-Tg

F

Interstitial fibrosis

(\%)

Sham TAC 1 week TAC 4 weeks TAC 8 weeks

Legend:

- WT (n=5-6)
- Cav-1-KO (n=5-6)
- eNOS-Tg (n=5-6)
Supplemental Figure VIII. Histological analysis of cardiac hypertrophy and fibrosis.  
**A**, Representative images of hematoxylin-eosin staining of the myocardium 8 weeks after TAC or sham operations.  **B**, Quantitative analysis of cross-sectional area of cardiomyocytes.  **C** and **E**, Representative images of Masson-trichrome staining for evaluation of perivascular (**C**) and interstitial (**E**) fibrosis in the hearts after 8 weeks of TAC or sham operations.  **D** and **F**, Quantitative analysis of perivascular (**D**) and interstitial (**F**) fibrosis.  n = 5-6 animals per group.  Scale bars = 100 μm.  Values are expressed as means±SEM.  *P<0.05, †P<0.01 vs. WT at each time point, ‡P<0.05, § P<0.01 vs. sham.
Supplemental Figure IX. Summary of the present study.
Summary of endothelium-dependent and -independent relaxations of small mesenteric arteries and coronary microvessels (upper panel) and the effects of cardiac pressure-overload (lower panel) are illustrated. Both Cav-1 deficiency and eNOS overexpression lead to over-activation of eNOS and excessive production of NO, causing disruption of the physiological balance between NO and EDH in microcirculations. As compared with WT mice, both Cav-1-KO and eNOS-Tg mice showed reduced survival after chronic cardiac pressure-overload induced by TAC, associated with accelerated left ventricular systolic dysfunction, reduced coronary flow reserve and enhanced myocardial hypoxia. Ca^{2+}/CaM denotes calcium/calmodulin; CFR, coronary flow reserve; Cu,Zn-SOD, copper, zinc-superoxide dismutase; K_{Ca}, calcium-activated potassium channels; L-Arg, L-arginine; L-Cit, L-citrulline; LV, left ventricular; PKG1α, protein kinase G1α; R, receptor; sGC, soluble guanylate cyclase; VSMC, vascular smooth muscle cell.
### Supplemental Table I. Baseline Characteristics and Echocardiographic Parameters.

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<th>WT (n)</th>
<th>Cav-1-KO (n)</th>
<th>eNOS-Tg (n)</th>
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<td><strong>Baseline characteristics</strong></td>
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<td>155±4 (17)†</td>
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<td>Heart weight/body weight, mg/g</td>
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<td>Systolic blood pressure, mmHg</td>
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<td>105±3 (6)</td>
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<td>Heart rate, bpm¶</td>
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<td>594±34 (6)</td>
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<td>Plasma nitrite/nitrate, μmol/L</td>
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<td>162.2±17.9 (17)†</td>
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<td><strong>Echocardiography</strong></td>
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<td>Heart rate, bpm#</td>
<td>473±6 (10)</td>
<td>492±7 (10)</td>
<td>492±9 (10)</td>
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<td>LVDd, mm</td>
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<td>3.54±0.07 (10)*</td>
<td>3.83±0.04 (10)</td>
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<td>LVDs, mm</td>
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<td>LVFS, %</td>
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<td>34.9±1.6 (10)</td>
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<td>IVST, mm</td>
<td>0.79±0.02 (10)</td>
<td>0.89±0.02 (10)†</td>
<td>0.76±0.03 (10)</td>
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<td>PWT, mm</td>
<td>0.73±0.01 (10)</td>
<td>0.87±0.02 (10)†</td>
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Results are expressed as means±SEM. bpm denotes beats per minute; Cav-1-KO, caveolin-1-knockout mice; eNOS-Tg, endothelium-specific endothelial nitric oxide synthase transgenic mice; IVST, interventricular septal thickness; LVDd, left ventricular (LV) end-diastolic diameter; LVDs, LV end-systolic diameter; LVFS, LV fractional shortening; PWT, posterior wall thickness; WT, wild-type mice. *P<0.05, †P<0.01 vs. WT. ¶Under conscious conditions. #Under light anesthesia with 0.5-0.8% isoflurane.
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<th>Agonist</th>
<th>Vessel (n)</th>
<th>Precon. (mN)</th>
<th>EC_{50} (-log mol/L)</th>
<th>Max. Relax. (%)</th>
<th>Precon. (mN)</th>
<th>EC_{50} (-log mol/L)</th>
<th>Max. Relax. (%)</th>
<th>Precon. (mN)</th>
<th>EC_{50} (-log mol/L)</th>
<th>Max. Relax. (%)</th>
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<td>Aorta (7)</td>
<td>8.4±0.4</td>
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<td>93.8±2.9</td>
<td>8.7±0.2</td>
<td>7.3±0.2†</td>
<td>72.2±4.6†</td>
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<td>6.7±0.2†</td>
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<td>NS-1619</td>
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<td>4.6±0.1</td>
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<td>5.4±0.1</td>
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<td>H_{2}O_{2}¶</td>
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<td>MA (6)</td>
<td>7.0±0.7</td>
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Results are expressed as means±SEM. Cav-1-KO indicates caveolin-1-knockout mice; eNOS-Tg, endothelium-specific endothelial nitric oxide synthase transgenic mice; EC_{50}, half-maximal effective concentration; H_{2}O_{2}, hydrogen peroxide; MA, mesenteric artery; Max. Relax., maximal relaxation; Precon., precontraction force to phenylephrine (10^{-6} mol/L); SNP, sodium nitroprusside; WT, wild-type mice. *P<0.05, †P<0.01 vs. WT. ¶In the presence indomethacin (10^{-5} mol/L) and L-NNA (10^{-4} mol/L).