Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Vascular Biology and Vascular Medicine

Mechanisms for Enhanced Endothelium-Derived Hyperpolarizing Factor-Mediated Responses in Microvessels in Mice

Junko Ohashi, MD, PhD; Ayuko Sawada, BSc; Sota Nakajima, MD, PhD; Kazuki Noda, MD; Aya Takaki, PhD; Hiroaki Shimokawa, MD, PhD

Background: Endothelium-derived relaxing factors play an important role in cardiovascular homeostasis. Among them, endothelium-derived hyperpolarizing factor (EDHF) is important especially in microcirculation. It has previously been demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) is an EDHF in animals and humans and that endothelial nitric oxide synthase (eNOS) plays diverse roles as a nitric oxide (NO) generating system in conduit arteries and as an EDHF/H_2O_2 generating system in microvessels. As compared with NO-mediated responses, those by EDHF are resistant to atherosclerosis, contributing to the maintenance of cardiovascular homeostasis. The aim of this study is to elucidate the molecular mechanisms for enhanced EDHF-mediated responses in microvessels.

Methods and Results: This study used male wild-type mice and caveolin-1-deficient mice (caveolin-1$^{-/-}$ mice). In the endothelium, eNOS was functionally suppressed in mesenteric arteries (microvessels) compared with the aorta (conduit arteries), for which Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) and caveolin-1 are involved, as EDHF-mediated responses were inhibited by STO-609 (an inhibitor of CaMKKβ) and in caveolin-1$^{-/-}$ mice, respectively. In vascular smooth muscle, relaxation responses to H_2O_2 were enhanced through a protein kinase G1α (PKG1α)-mediated mechanism in mesenteric arteries compared with the aorta, as they were inhibited by Rp-8-Br-cGMPS (an inhibitor of PKG1α).

Conclusions: These results indicate that CaMKKβ, caveolin-1, and PKG1α are substantially involved in the mechanisms for the enhanced EDHF-mediated responses in microvessels in mice. (*Circ J* 2012; **76:** 1768–1779)

Key Words: Endothelial nitric oxide synthase; Endothelium-derived hyperpolarizing factor; Microveesels; Nitric oxide

The endothelium plays an important role in maintaining cardiovascular homeostasis by synthesizing and releasing several vasodilators, including prostacyclin (PGI_2), nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF).[1–3] EDHF-mediated responses are classically defined as the endothelium-dependent responses (relaxations and hyperpolarizations) after the blockade of the synthesis of PGI_2 and NO.[4–6] It is widely accepted that EDHF plays an important role in modulating vascular tone, especially in microvessels, among all species and tissues studied.[7,8] As compared with NO-mediated responses, EDHF-mediated responses are relatively resistant to atherosclerosis and thus are considered as a back-up system for NO-mediated responses.[5,8] Indeed, in patients with coronary artery disease, endothelium-dependent vasodilator functions are easily deteriorated in epicardial coronary arteries but are fairly preserved in coronary microvessels.[8]

Editorial p1599

For the nature of EDHF, several candidates have been proposed, including epoxyeicosatrienoic acids,[9,10] K^+ ions,[11,12] gap junctions,[13,14] and, as we have previously identified, hydrogen peroxide (H_2O_2).[15–17] We have demonstrated that endothelium-derived H_2O_2 is an EDHF in mouse[15] and human[16] mesenteric arteries and porcine coronary microvessels.[17] Subsequently, other investigators have confirmed the importance of H_2O_2 as an EDHF in human[18] and canine[19,20] coronary microvessels, and piglet pial arteries.[21] We also have demonstrated that endothelial NO synthase (eNOS)-derived superoxide anions are a major source of EDHF/H_2O_2,[15,22,23] where copper and zinc superoxide dismutase (Cu,Zn-SOD) plays an important role

Received February 13, 2012; revised manuscript received February 26, 2012; accepted February 27, 2012; released online March 30, 2012
Time for primary review: 11 days
Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
The Guest Editor for this article was Kenichi Hirata, MD.
Mailing address: Hiroaki Shimokawa, MD, PhD, Professor and Chairman, 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
ISSN-1346-9843 doi:10.1253/circj.CJ-12-0197

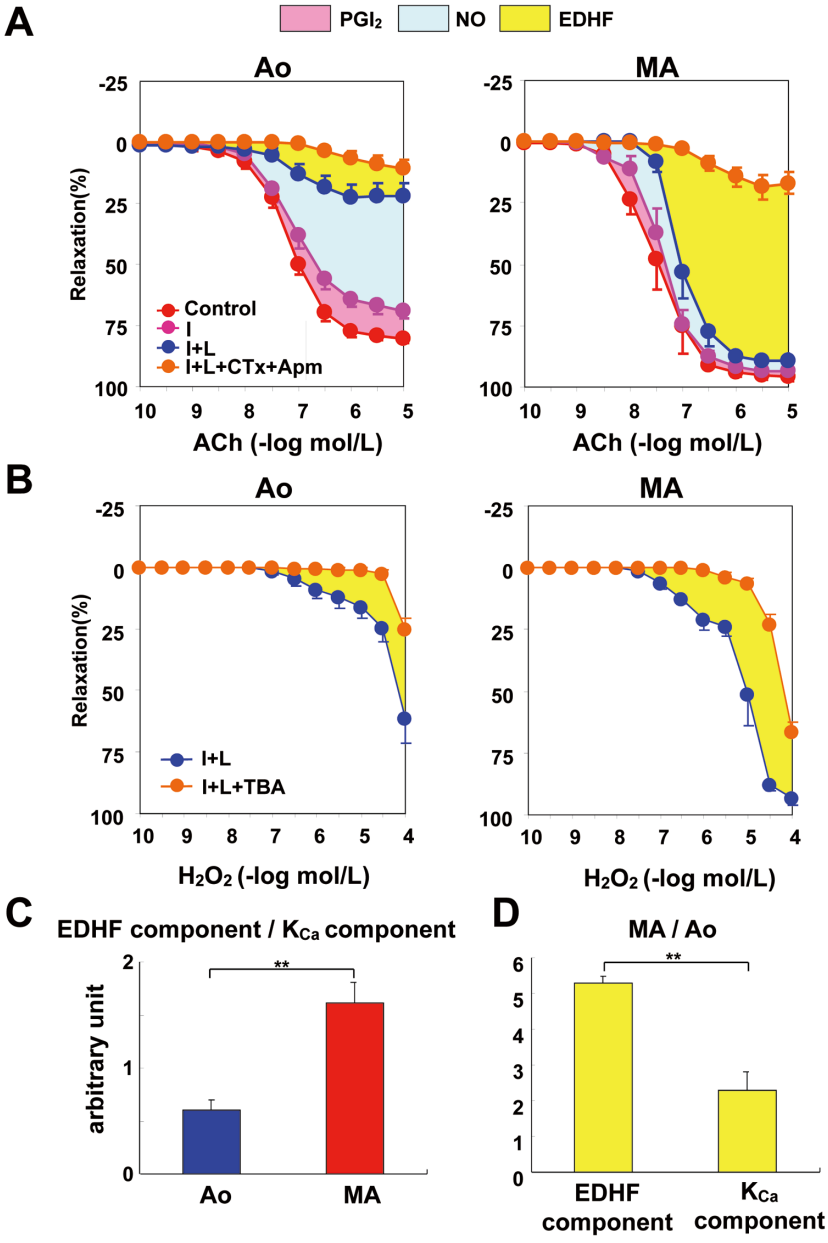

Figure 1. Relative contribution of the endothelium and VSMC to EDHF-mediated relaxations in mice. (**A**) In the aorta (Ao), endothelium-dependent relaxations to ACh were markedly inhibited by L-NNA, whereas in mesenteric arteries (MA), they were highly resistant to indomethacin, L-NNA or their combination, but were markedly inhibited by a combination of charybdotoxin and apamin (n=6). I, indomethacin; L, L-NNA; CTx, charybdotoxin; Apm, apamin. (**B**) The extent of relaxations to exogenous H_2O_2 and the inhibitory effects of TBA (1 mmol/L, 30 min) were both greater in MA than in Ao (n=4). (**C**) The ratio of EDHF-mediated relaxations (to ACh) to direct VSMC relaxations (to exogeneous H_2O_2) was significantly greater in MA than in Ao. (**D**) The ratio of relaxations in MA to those in Ao was significantly greater for EDHF-mediated relaxations than for K_{Ca}-mediated (TBA-sensitive) relaxations. EDHF-mediated and exogenous H_2O_2 -induced relaxations were examined in the same animals (n=4 each). **P<0.01. VSMC, vascular smooth muscle; EDHF, endothelium-derived hyperpolarizing factor; L-NNA, N^{ω}-nitro-L-arginine; H_2O_2, hydrogen peroxide.

to dismutate eNOS-derived superoxide anions to synthesize EDHF/H_2O_2 in animals and humans.[24,25] There are several intracellular sources of superoxide anions other than NOSs, including nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, xanthine oxidase, lipoxygenase, and the mitochondrial electron transport chain, but they are not involved in EDHF/H_2O_2 responses in mice.[23] In vivo, we have elucidated that EDHF/H_2O_2 plays an important role in coronary microcirculation, including coronary autoregulation,[19] cardiovascular protection against myocardial ischemia-perfusion injury[20] and metabolic coronary vasodilatation in canine coronary microcirculation in vivo.[26]

Although it has been demonstrated that EDHF-mediated responses are dominant in microvessels,[7,8] the molecular mechanisms remain to be elucidated. It would provide important clues for the novel strategy for vascular protection to elucidate the molecular mechanisms involved for the enhanced EDHF-mediated responses in microvessels. In the present study, we thus addressed this important issue in mice.

Methods

Animal and Tissue Preparation

This study was reviewed and approved by the Committee on Ethics of Animal Experiments of the Tohoku University. Male C57BL/6 wild-type (WT) mice and caveolin-1-deficient (Cav-$1^{-/-}$) mice that were 12–16 weeks of age were used. Cav-$1^{-/-}$ mice were derived from breeding pairs of heterozygous (Cav-$1^{+/-}$) mice (Jackson Laboratory, Barharbor, ME, USA) and were maintained in the Laboratory of Animal Experiments in the Tohoku University. The Cav-$1^{-/-}$ mice were backcrossed to C57BL/6 mice over 10 generations and thus C57BL/6 mice

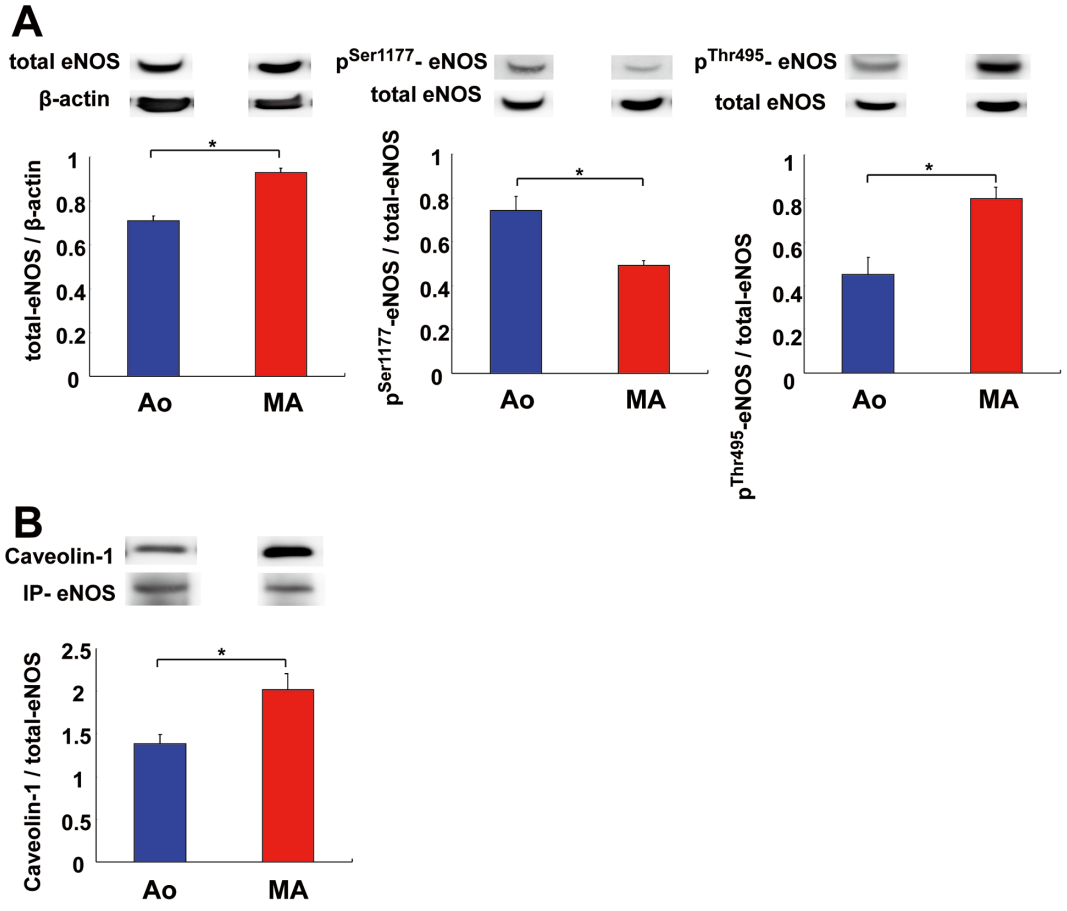

Figure 2. eNOS is functionally suppressed in mesenteric arteries in mice. (**A**) The extent of eNOS phosphorylation at Ser1177 was significantly suppressed and that at Thr495 was significantly greater in mesenteric arteries (MA) than in the aorta (Ao) (n=3 each). (**B**) Immunoblot with an anti-caveolin-1 antibody of the extracts immunoprecipitated with anti-eNOS antibody showed that the extent of eNOS coupled with caveolin-1 was significantly greater in MA than in Ao (n=3). *P<0.05. eNOS, endothelial nitric oxide synthase.

were used as a WT control. The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and the aorta and small mesenteric arteries (200–250 μm in diameter) were excised.[15,22,24] To examine endothelium-independent responses, some arterial rings were stripped of the endothelium by gently rubbing the inner surface with cotton string.[15,22,24] To extract protein for Western blot analysis, we isolated the aorta and mesenteric arteries from any connective tissues.

Organ Chamber Experiments

Experiments were performed in 37°C Krebs solution bubbled with 95% O_2 and 5% CO_2. Isometric tension was recorded in isolated arterial rings contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (3–10 μmol/L).[15,24] The extent of the contraction was adjusted to 50–70% of the contractions induced by 60 mmol/L potassium chloride (KCl). Endothelium-dependent relaxations to acetylcholine (ACh) and endothelium-independent relaxations to sodium nitroprusside (SNP), NS-1619 [a direct opener of calcium-activated potassium (K_{Ca}) channels], or exogenous H_2O_2 were examined. The contributions of vasodilators PGI_2, NO, and EDHF to ACh-induced endothelium-dependent relaxations were determined by the inhibitory effect of indomethacin (10 μmol/L), N^{ω}-nitro-L-arginine (L-NNA, 100 μmol/L), and a combination of charybdotoxin (100 μmol/L, an inhibitor of large and intermediate-conductance K_{Ca} channels) and apamin (1 μmol/L, an inhibitor of small-conductance K_{Ca} channels), respectively.[14] Indomethacin, L-NNA, charybdotoxin and apamin were applied to organ chambers 30 min before precontraction with $PGF_{2\alpha}$. To examine the relative contribution of endothelium-derived relaxing factors, the NO-mediated response was evaluated by the area under the response curve that was inhibited by L-NNA in the presence of indomethacin and EDHF-mediated response by the area under the response curve that was inhibited by a combination of charybdotoxin and apamin in the presence of indomethacin and L-NNA.[15,22–24] Quantitative analysis of exogenous H_2O_2-induced relaxations in endothelium-denuded arterial rings was done by the area under the response curve that was inhibited by tetrabutyl-ammonium (TBA, 1 mmol/L, a non-specific inhibitor of K_{Ca} channels), which was applied to the organ chamber 30 min before precontraction with $PGF_{2\alpha}$.

Additional experiments were performed using the following inhibitors: STO-609 [5 μmol/L, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ),[27] compound C [10 μmol/L, an inhibitor of adenosine monophosphate-activated protein kinase (AMPK)],[28] wortmannin [100 nmol/L,

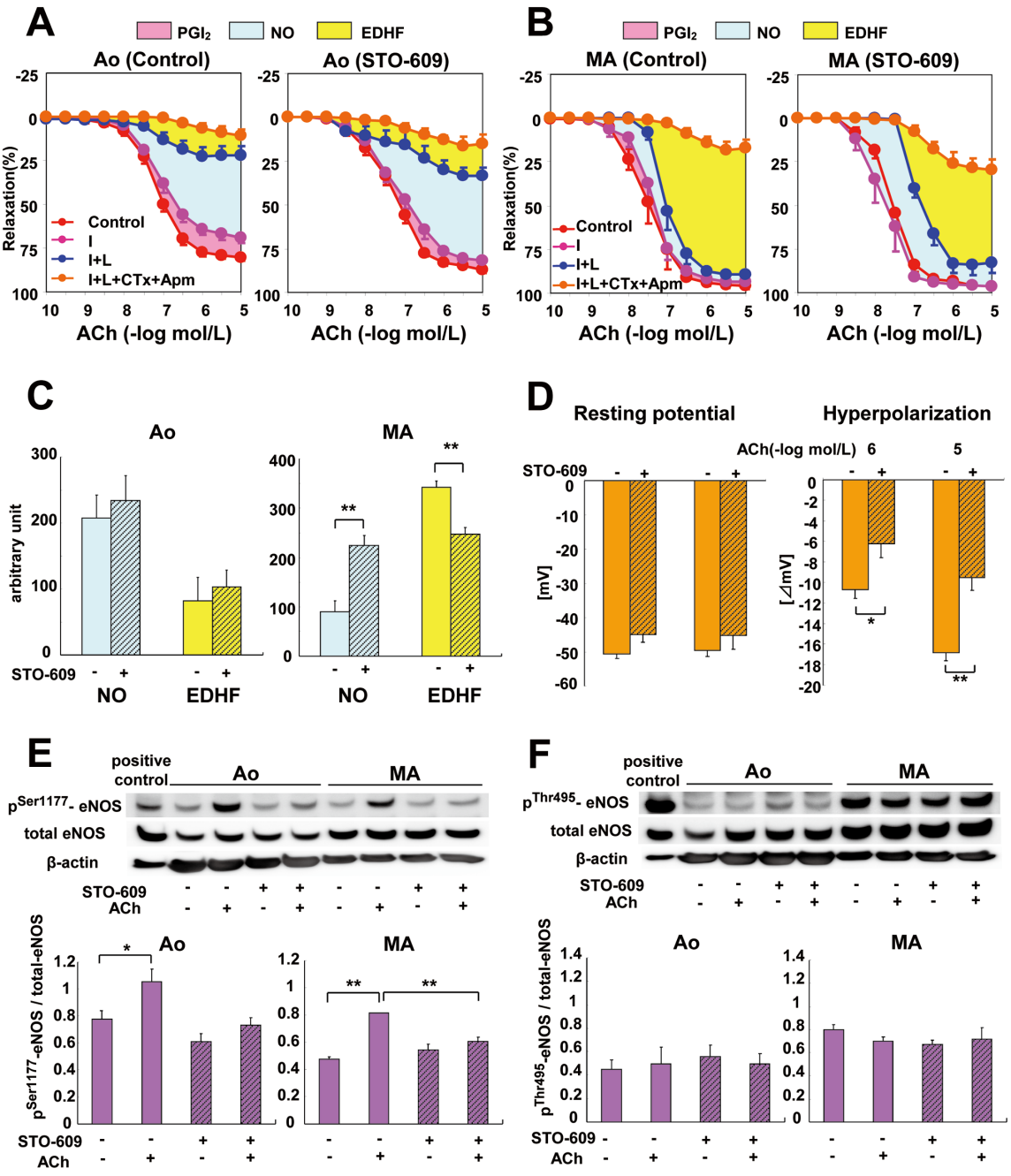

Figure 3. Involvement of CaMKKβ in EDHF-mediated relaxations. (**A**,**B**) Endothelium-dependent relaxations to ACh with or without preincubation with STO-609 (5 mmol/L, 60 min) in the aorta (Ao) and mesenteric arteries (MA) (n=6 each). STO-609 had no effects in the Ao but significantly inhibited EDHF-mediated responses in MA. (**C**) Quantitative analysis of the relative contribution of NO and EDHF to the endothelium-dependent relaxations to ACh with or without preincubation with STO-609 (5 mmol/L, 60 min) in the Ao and MA (n=6 each). STO-609 significantly inhibited EDHF-mediated relaxations and significantly enhanced NO-mediated relaxations in MA but had no effects in the Ao. (**D**) Endothelium-dependent hyperpolarizations to ACh of mesenteric arteries with or without preincubation with STO-609 (5 mmol/L, 60 min) (n=6 each). STO-609 significantly inhibited EDHF-mediated hyperpolarizations in MA but not in the Ao. (**E**) The extent of eNOS phosphorylation at Ser1177 was significantly enhanced in response to ACh in both MA and the Ao, which was inhibited by STO-609 (5 mmol/L, 60 min) in MA but not in the Ao (n=3 each). (**F**) The extent of eNOS phosphorylation at Thr495 was unchanged by STO-609 (5 mmol/L, 60 min) in MA or the Ao (n=3 each). *P<0.05, **P<0.01. CaMKKβ, Ca^{2+}/calmodulin-dependent protein kinase kinase β; EDHF, endothelium-derived hyperpolarizing factor; ACh, acetylcholine; NO, nitric oxide.

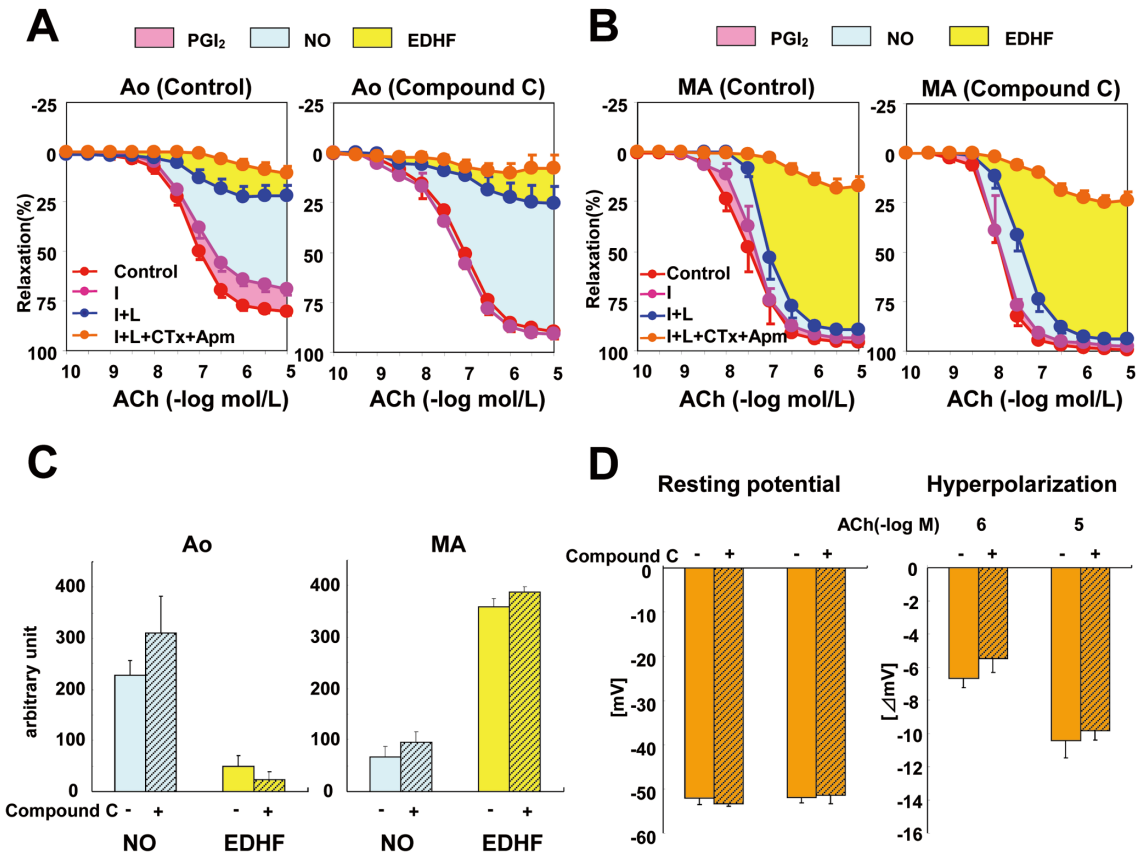

Figure 4. No involvement of AMPK in EDHF-mediated relaxations (**A**,**B**). Endothelium-dependent relaxations to ACh with or without preincubation with compound C (10 mmol/L, 30 min) in the aorta (Ao) and mesenteric arteries (MA) (control n=6, compound C n=4). (**C**) Quantitative analysis of the relative contribution of NO and EDHF to the endothelium-dependent relaxations to ACh with or without preincubation with compound C (10 mmol/L, 30 min) in the Ao and MA (control n=6, compound C n=4). (**D**) Endothelium-dependent hyperpolarizations to ACh of mesenteric arteries with or without preincubation with compound C (10 mmol/L, 60 min) (n=6 each). Compound C had no effects on EDHF-mediated relaxations or hyperpolarizations to ACh in both-sized arteries. AMPK, adenosine monophosphate-activated protein kinase; EDHF, endothelium-derived hyperpolarizing factor; ACh, acetylcholine; NO, nitric oxide.

an inhibitor of phosphatidylinositol-3-kinase (PI3K)][29] and Rp-8-bromo-guanosine 3',5'-monophosphothionate [Rp-8-Br-cGMPS, 200 μmol/L, an inhibitor of protein kinase G1α (PKG1α).[30] Compound C, wortmannin and Rp-8-Br-cGMPS were applied to organ chambers 30 min before precontraction with $PGF_{2\alpha}$, and STO-609 was applied 60 min before.

Electrophysiological Experiments

Electrophysiological experiments were performed with isolated small mesenteric arteries. The rings of small mesenteric arteries were placed in experimental chambers perfused with 37°C Krebs solution containing indomethacin (10 μmol/L) and L-NNA (100 μmol/L) bubbled with 95% O_2 and 5% CO_2. A fine glass capillary microelectrode was impaled into the smooth muscle cell from the adventitial side of mesenteric arteries, and changes in membrane potentials produced by ACh (10 and 100 μmol/L) were continuously recorded.[15,24]

Western Blot Analysis

The extract protein from isolated aorta and mesenteric arteries was incubated in Krebs solution for 60 min (the aorta 20 μg, mesenteric arteries 10 μg) and was then loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot analysis was performed using antibodies that specially recognize protein, including eNOS, Ser1177-phosphorylated or Thr495-phosphorylated eNOS, caveolin-1 and β-actin (internal control). Immunoprecipitation of the eNOS protein was performed using μMACS™ and MultiMACS™ Protein G Kits (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).[31,32]

Statistical Analysis

Data are shown as mean±SEM. A dose-response curve was analyzed by using a 2-way ANOVA followed by Scheffe's post-hoc test for multiple comparisons. Other values were analyzed by paired and unpaired t-tests or a 1-way ANOVA. A $P<0.05$ was considered to be statistically significant.

Results

Relative Contribution of the Endothelium and Vascular Smooth Muscle to Enhanced EDHF-Mediated Responses in Microvessels

We first examined to what extent the endothelium and vascu-

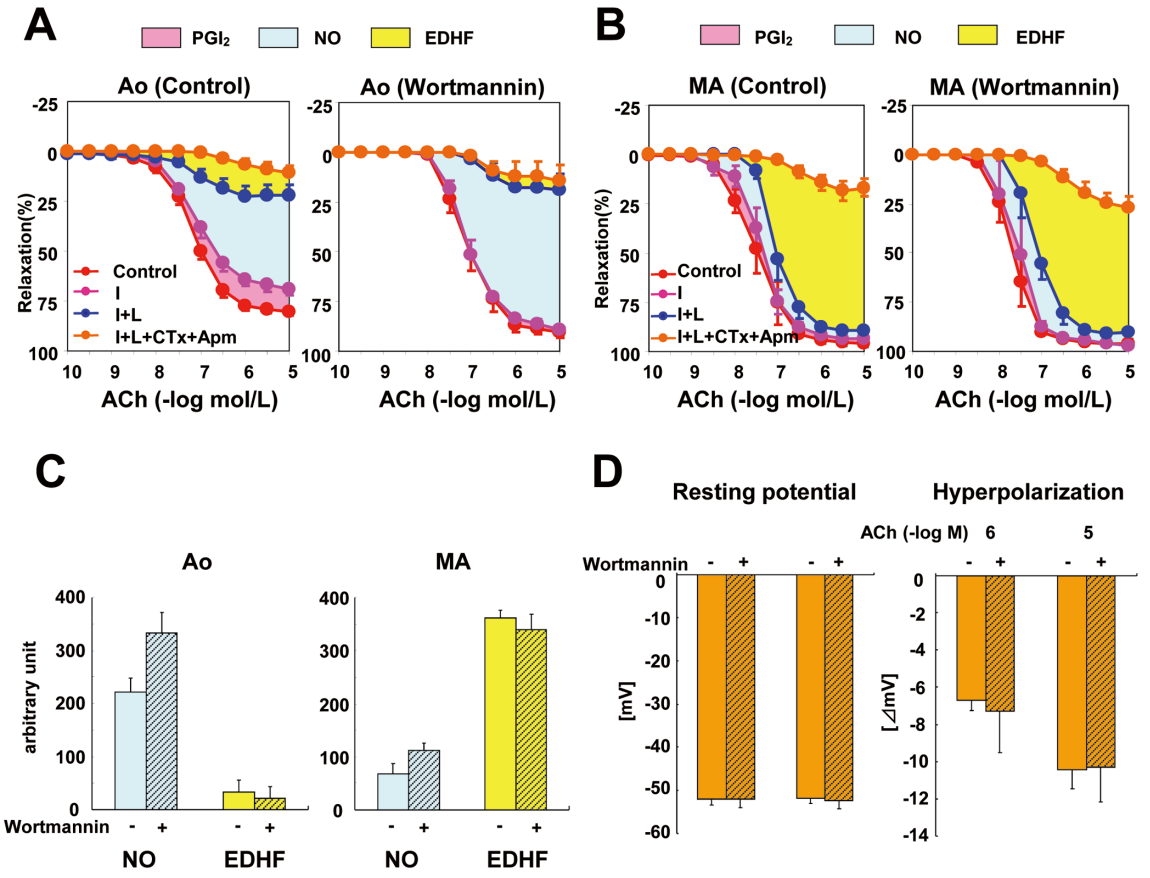

Figure 5. No involvement of PI3K in EDHF-mediated relaxations. (**A**,**B**) Endothelium-dependent relaxation to ACh with or without preincubation with wortmannin (100 nmol/L, 30 min) in the aorta (Ao) and mesenteric arteries (MA) (control n=6, wortmannin n=4). (**C**) Quantitative analysis of the relative contribution of NO and EDHF to the endothelium-dependent relaxations to ACh with or without preincubation with wortmannin (100 nmol/L, 30 min) in the Ao and MA (control n=6, wortmannin n=4). (**D**) Endothelium-dependent hyperpolarizations to ACh of mesenteric arteries with or without preincubation with wortmannin (100 nmol/L, 30 min) (control n=6, wortmannin n=4). Wortmannin had no effects on EDHF-mediated relaxations or hyperpolarizations to ACh in both-sized arteries. EDHF, endothelium-derived hyperpolarizing factor; ACh, acetylcholine; NO, nitric oxide.

lar smooth muscle cells (VSMC) contribute to the enhanced EDHF-mediated responses in microvessels in WT mice. As we have repeatedly reported,[15,16,22] endothelium-dependent relaxations of the aorta to ACh were markedly inhibited by L-NNA (a NOS inhibitor) in the presence of indomethacin (a PGI_2 synthesis inhibitor), whereas the relaxation responses of mesenteric arteries to ACh were resistant to indomethacin, L-NNA or their combination, but were markedly inhibited by a combination of charybdotoxin (an inhibitor of large- and intermediate-conductance K_{Ca} channels) and apamin (an inhibitor of small-conductance K_{Ca} channels), indicating a primary role of EDHF (**Figure 1A**). These results confirmed our notion that NO and EDHF play an important role in endothelium-dependent relaxations in the conduit arteries (the aorta) and microvessels (mesenteric arteries), respectively.[7,22]

We then examined to what extent VSMC are involved in the enhanced EDHF-mediated responses of microvessels to EDHF/H_2O_2. Exogenous H_2O_2 (10^{-10}–10^{-4} mol/L) caused concentration-dependent direct relaxations in rings without endothelium (in the presence of indomethacin and L-NNA), which were enhanced in mesenteric arteries as compared with the aorta (**Figure 1B**). In addition, the H_2O_2-mediated direct relaxations were markedly inhibited by TBA (a non-specific inhibitor of K_{Ca} channels), which was consistent with the definition of H_2O_2 as an EDHF (**Figure 1B**).

When the EDHF-mediated component in endothelium-dependent relaxations (contribution of the endothelium) was normalized by exogenous H_2O_2-mediated relaxations (contribution of VSMC), it was evident that the role of the endothelium was greater in mesenteric arteries compared with the aorta (**Figure 1C**). This was also the case in mesenteric arteries/the aorta ratio of EDHF- and K_{Ca}-mediated responses (**Figure 1D**). These results indicate that both the endothelium and VSMC contribute to the enhanced EDHF-mediated relaxations; the endothelium to a greater extent than VSMC.

Endothelial Level (1): eNOS Is Functionally Suppressed in Mesenteric Arteries

Then, we examined the molecular mechanisms for the divergent roles of eNOS between the aorta and mesenteric arteries. We have previously demonstrated that eNOS is not pathologically uncoupled in mouse mesenteric arteries.[22] We thus examined first whether eNOS activity is altered in mouse mesenteric arteries. Among the several phosphorylation sites

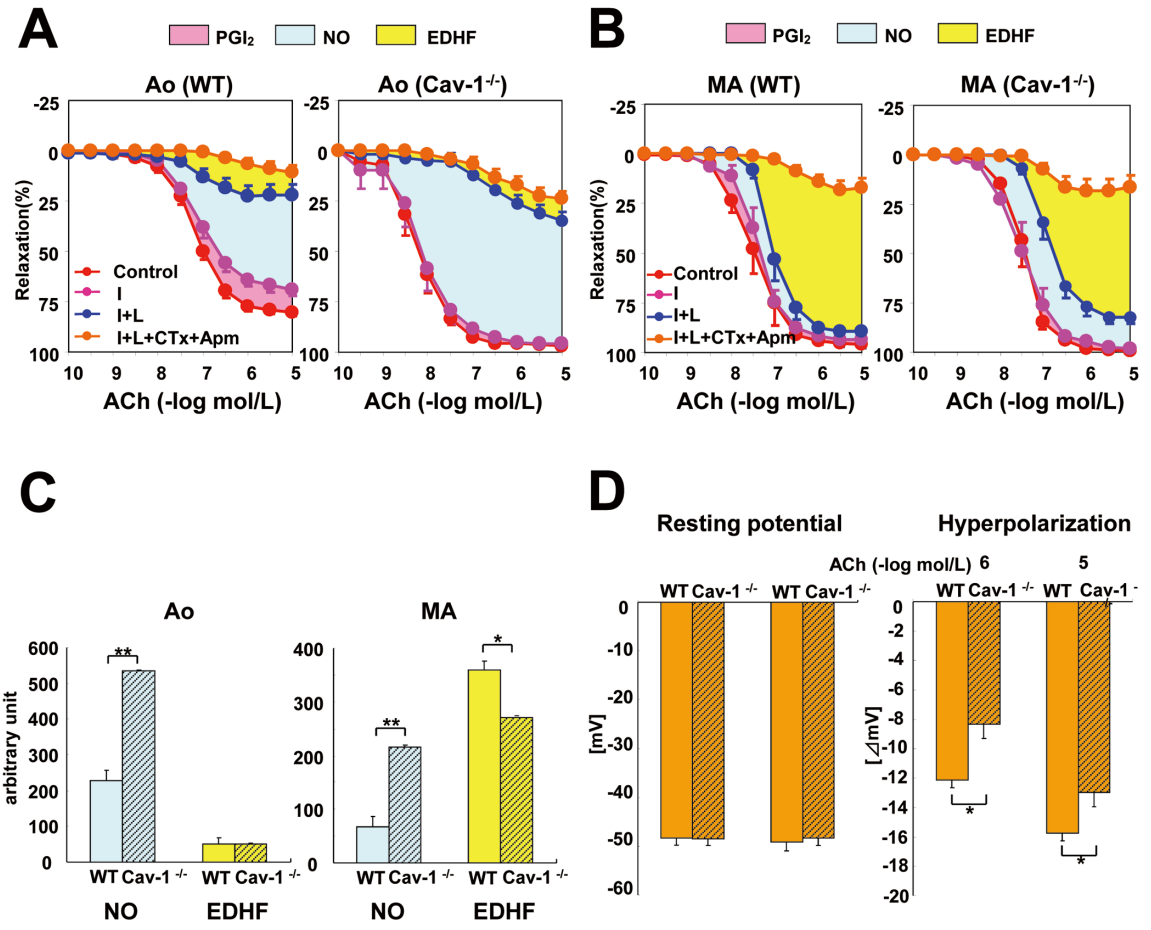

Figure 6. Involvement of caveolin-1 in EDHF-mediated relaxations. (**A**,**B**) Endothelium-dependent relaxation to ACh of the aorta (Ao) and mesenteric arteries (MA) in WT and Cav-1$^{-/-}$ mice (WT n=6, Cav-1$^{-/-}$ n=5). (**C**) Quantitative analysis of the relative contribution of NO and EDHF to the endothelium-dependent relaxations to ACh in the Ao and MA (WT n=6, Cav-1$^{-/-}$ n=5). (**D**) Endothelium-dependent hyperpolarizations to ACh of mesenteric arteries from WT mice and Cav-1$^{-/-}$ mice (WT n=6, Cav-1$^{-/-}$ n=5). In Cav-1$^{-/-}$ mice, EDHF-mediated relaxations and endothelium-dependent hyperpolarizations to ACh were significantly reduced in MA, whereas NO-mediated relaxations were significantly enhanced in both the Ao and MA. *P<0.05, **P<0.01. ACh, acetylcholine; NO, nitric oxide; EDHF, endothelium-derived hyperpolarizing factor; WT, wild-type; Cav-1$^{-/-}$, caveolin-1-deficient mice.

of eNOS, it is well known that Ser1177 is an important stimulatory site and that Thr495 is an important inhibitory site in mice and humans.[33] Western blotting experiments demonstrated that the extent of eNOS phosphorylation at Ser1177 was significantly reduced and that at Thr495 was significantly greater in mesenteric arteries than in the aorta (**Figure 2A**). Furthermore, immunoprecipitation experiments demonstrated that the extent of eNOS coupling with caveolin-1 was significantly greater in mesenteric arteries than in the aorta (**Figure 2B**). These results suggest that eNOS is functionally suppressed in mesenteric arteries compared with the aorta even under physiological conditions, at least in part, by the caveolin-1-dependent mechanism.

Endothelial Level (2): Role of CaMKKβ for Enhanced EDHF-Mediated Responses in Mesenteric Arteries

It has been demonstrated that multiple intracellular mechanisms are involved for eNOS activation, including CaMKKβ, AMPK, PI3K, Akt, and caveolin-1.[27] Thus, we examined which mechanism is involved in the functional eNOS suppression in mouse mesenteric arteries. We first examined the inhibitory effects of STO-609, an inhibitor of CAMKKβ.[27,34] Although STO-609 (5 μmol/L, 60 min) had no effects on contractions to KCl or endothelium-independent relaxations to SNP (**Figures S1A–C**), it significantly inhibited EDHF-mediated relaxations and hyperpolarizations to ACh and significantly enhanced NO-mediated relaxations to ACh in mesenteric arteries, whereas it had no effects in the aorta (**Figures 3A–D**). Consistent with the results of organ chamber experiments, the extent of eNOS phosphorylation at Ser1177 in response to ACh was significantly enhanced both in the aorta and mesenteric arteries, which was inhibited by STO-609 in mesenteric arteries more than in the aorta (**Figure 3E**). In contrast, the extent of eNOS phosphorylation at Thr495 was unchanged by STO-609 in both-sized arteries (**Figure 3F**). These results suggest the involvement of CaMKKβ in the enhanced EDHF-mediated responses in mouse mesenteric arteries through eNOS phosphorylation at Ser1177.

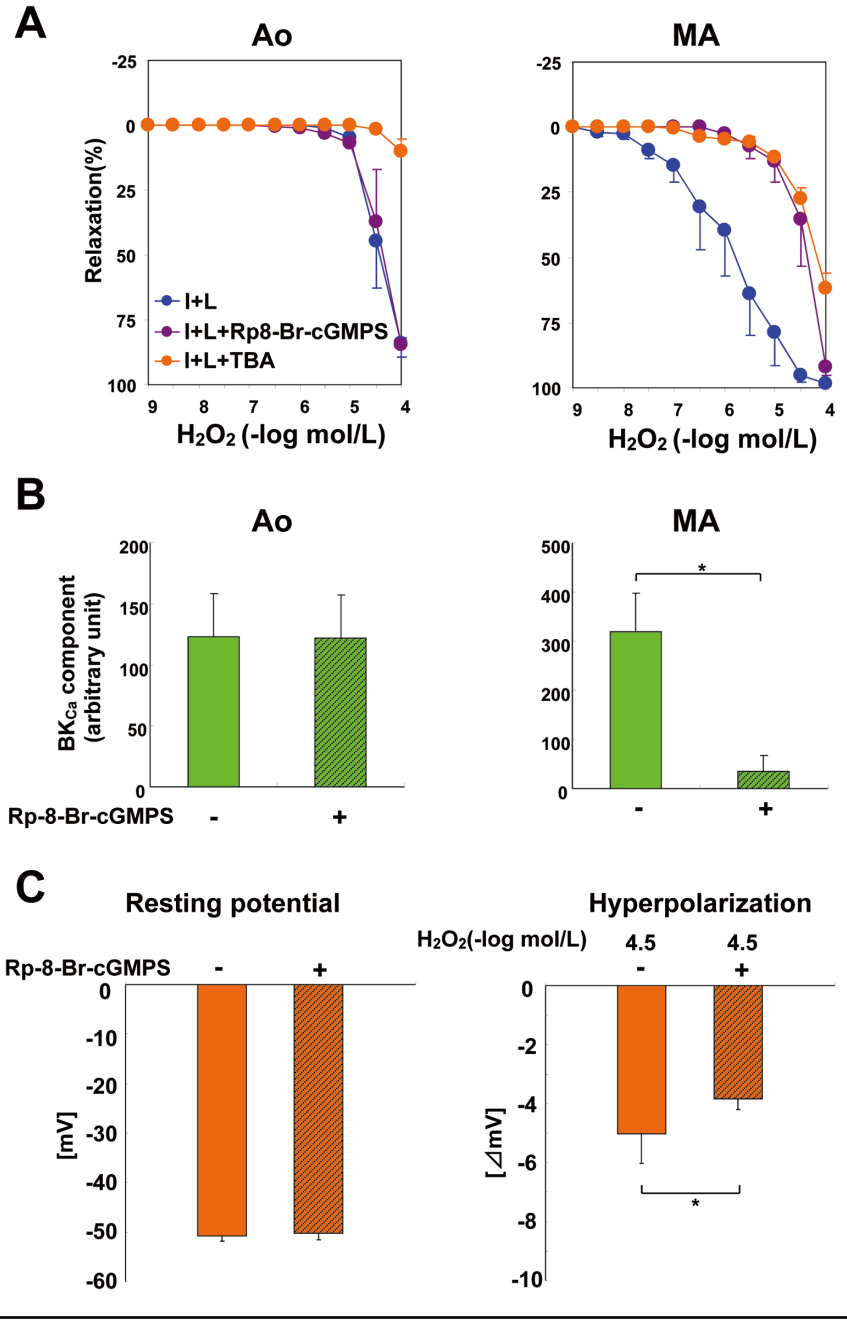

Figure 7. Involvement of PKG1a in H_2O_2-induced direct relaxations of VSMC in mesenteric arteries. (**A**) Exogenous H_2O_2-induced direct relaxations of the endothelium-denuded aorta (Ao) and mesenteric arteries (MA) without or with preincubation with Rp-8-Br-cGMPS (200 mmol/L, 30 min) or TBA (1 mmol/L, 30 min) (n=4 each). (**B**) Quantitative analysis of exogenous H_2O_2-induced relaxations in the endothelium-denuded Ao and MA without or with preincubation with Rp-8-Br-cGMPS (200 mmol/L, 30 min) (n=4 each). (**C**) Exogenous H_2O_2-induced hyperpolarizations in endothelium-denuded MA without or with preincubation with Rp-8-Br-cGMPS (200 mmol/L, 30 min) or TBA (1 mmol/L, 30 min) (n=3 each). Rp-8-Br-cGMPS significantly inhibited exogenous H_2O_2-induced relaxations and hyperpolarizations in MA, but not in the Ao. *P<0.05. H_2O_2, hydrogen peroxide; VSMC, vascular smooth muscle; TBA, tetrabutyl-ammonium.

Endothelial Level (3): Role of AMPK for Enhanced EDHF-Mediated Responses in Mesenteric Arteries

We then examined the role of AMPK by using compound C, an inhibitor of AMPK.[27,28] Compound C (10 μmol/L, 30 min) had no effects on EDHF-mediated relaxations or hyperpolarizations to ACh of the aorta or mesenteric arteries (**Figures 4A–D**), whereas it attenuated contractions of mesenteric arteries to KCl in a concentration-dependent manner (**Figure S2A**). Compound C had no effects on endothelium-independent relaxations to SNP or NS-1619, an opener of K_{Ca} channels, in both-sized arteries (**Figures S2B–C**). These results suggest no involvement of AMPK in EDHF-mediated relaxations in mouse mesenteric arteries.

Endothelial Level (4): Role of PI3K for Enhanced EDHF-Mediated Responses in Mesenteric Arteries

We examined the role of PI3K by using its inhibitor, wortmannin.[29] Wortmannin (100 nmol/L, 30 min) had no effects on EDHF-mediated relaxations or hyperpolarizations to ACh in the aorta or mesenteric arteries (**Figures 5A–D**), whereas it attenuated contractions of the aorta and mesenteric arteries in a concentration-dependent manner (**Figure S3A**). Wortmannin had no effects on endothelium-independent relaxations to SNP or NS-1619 in both-sized arteries (**Figures S3B–C**). These results suggest no involvement of PI3K in EDHF-mediated relaxations in mouse mesenteric arteries.

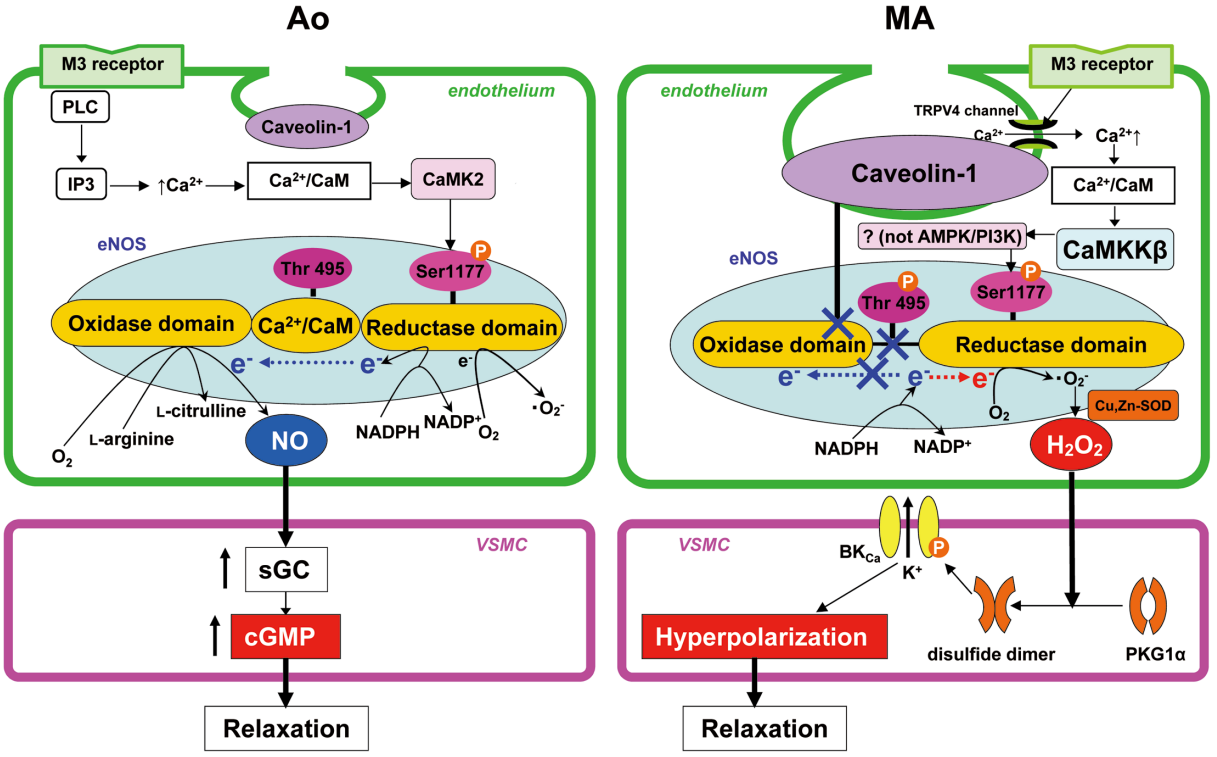

Figure 8. Summary of the present study. Multiple mechanisms are involved in the enhanced EDHF-mediated responses in microvessels, including eNOS suppression mainly by CaMKKβ and caveolin-1 at the endothelial levels and enhanced vascular responsiveness to EDHF/H_2O_2 by PKG1α at the vascular smooth muscle level. The extent of the relative contribution of the endothelium and vascular smooth muscle (VSMC) to the enhanced EDHF-mediated responses is greater for former than for the latter. M3 receptor, muscarinic M3 receptor; Gq, Gq protein; IP3, inositol triphosphate; CaM, calmodulin; $\cdot O_2^-$, superoxide anions; CaMK2, calcium/calmodulin-dependent protein kinase 2; CaMKKβ, Ca^{2+}/calmodulin-dependent protein kinase kinase β; eNOS, endothelial nitric oxide synthase; EDHF, endothelium-derived hyperpolarizing factor; H_2O_2, hydrogen peroxide.

Endothelial Level (5): Role of Cav-1 for Enhanced EDHF-Mediated Responses in Mesenteric Arteries

Systolic blood pressure in Cav-$1^{-/-}$ mice tended to be lower as compared with WT mice (105.6±4.3 vs. 120.0±4.2 mmHg, P=0.05). In Cav-$1^{-/-}$ mice, as compared with WT mice, NO-mediated relaxations were significantly enhanced in the aorta and mesenteric arteries, and EDHF-mediated relaxations were significantly reduced in mesenteric arteries (Figures 6A–C). Consistent with the results of organ chamber experiments, endothelium-dependent hyperpolarizations to ACh in mesenteric arteries from Cav-$1^{-/-}$ mice were significantly attenuated compared with WT mice (Figure 6D). Endothelium-independent relaxations to SNP in both the aorta and mesenteric arteries were slight but significantly enhanced in Cav-$1^{-/-}$ mice compared with WT mice, whereas those to NS-1619 were comparable (Figures S4A,B). These results suggest the involvement of caveolin-1 in the enhanced EDHF-mediated relaxations in mesenteric arteries.

VSMC Level: Role of PKG1α for Enhanced EDHF-Mediated Responses in Mesenteric Arteries

It has recently been reported that PKG1α mediates H_2O_2-induced relaxation of VSMC, in which phosphorylation of large-conductance K_{Ca} (BK_{Ca}) channels is involved.[30,35] We used Rp-8-Br-cGMPS as an inhibitor of PKG1α[30] Rp-8-Br-cGMPS (200 μmol/L, 30 min) significantly inhibited exogenous H_2O_2-induced direct relaxations and hyperpolarizations of mesenteric arteries, but did not inhibit those of the aorta (Figures 7A–C). These results suggest an involvement of a PKG1α-mediated mechanism in VSMC for the enhanced EDHF/H_2O_2-mediated relaxations in mesenteric arteries.

Discussion

The major findings of the present study are as follows: (1) both the endothelium and VSMC contribute to the enhanced EDHF-mediated responses in mesenteric arteries, although the contribution of the endothelium is much greater than that of VSMC; (2) at the endothelial level, eNOS is functionally suppressed in mesenteric arteries compared with the aorta, for which mechanisms mediated by CaMKKβ and caveolin-1 might be involved; (3) genetic disruption of caveolin-1 reduces EDHF-mediated responses; and (4) at the VSMC level, the relaxation response to EDHF/H_2O_2 is enhanced by PKG1α-mediated mechanisms (Figure 8). To the best of our knowledge, this is the first report that demonstrates the molecular mechanisms for the enhanced EDHF-mediated responses in microvessels. These mechanisms might be important to develop a new strategy for cardiovascular protection, as EDHF-mediated responses are relatively resistant as compared with NO-mediated responses.[5,8]

Relative Contribution of the Endothelium and VSMC to the Enhanced EDHF-Mediated Responses in Microvessels

We have previously demonstrated that microvascular endothelial cells synthesize and release EDHF/H_2O_2 in response to ACh, bradykinin and substance P, and that exogenous H_2O_2 induces direct relaxations and hyperpolarizations of VSMC in microvessels.[15,17,22] Furthermore, we have previously confirmed that the VSMC responses to SNP and NS-1619 are comparable between the aorta and mesenteric arteries.[22] In the present study, we examined for the first time the relative contribution of the endothelium and VSMC to the enhanced EDHF-mediated responses in mouse mesenteric arteries. The results showed that both the endothelium and VSMC contribute to the enhanced EDHF-mediated responses, but to a great extent by the former than by the latter. Although we examined the relative contribution of the endothelium and VSMC with regard to relaxation responses in vitro, a more precise comparison would be the direct measurement of H_2O_2 synthesis by the endothelium between the aorta and mesenteric arteries. However, we found that it is technically difficult to quantify endothelial H_2O_2 production in the aorta and therefore we compared the relative contribution of the endothelium and VSMC to the enhanced EDHF-mediated responses functionally by the relaxation responses in vitro. We then examined the role of the endothelium and VSMC separately in the following experiments.

Functional Suppression of eNOS in Mesenteric Arteries

In order to elucidate the molecular mechanisms for the enhanced EDHF-mediated response in microvessels, we first examined the possible difference in eNOS activity between the aorta and mesenteric arteries; we have demonstrated that eNOS plays a pivotal role not only for NO-mediated responses in the aorta but also for EDHF-mediated responses in mesenteric arteries.[15,22,23] We found that among the eNOS phosphorylation sites,[33] phosphorylation at Ser1177 (stimulatory site) is significantly reduced and that at Thr495 (inhibitory site), it is significantly enhanced in mesenteric arteries compared with the aorta under basal conditions, indicating that eNOS is functionally suppressed in mesenteric arteries. In the present study, we did not examine the expression or affinity of endothelial muscarinic receptors, however, it is widely known that in both the aorta and mesenteric arteries, ACh couples to the muscarinic M3 receptor,[36,37] which is followed by an increase in [Ca^{2+}]i and Ca^{2+}/CaM complex formation.[37] This pathway is commonly used for the synthesis of both NO and EDHF in response to ACh.[6] In the present study, ACh markedly enhanced eNOS phosphorylation at Ser1177 but not at Thr495 in mesenteric arteries. Thus, we then examined the intracellular signaling pathway for eNOS activation in the process between Ca^{2+}/CaM and eNOS phosphorylation at Ser1177, including CaMKKβ, AMPK, PI3K, and caveolin-1.

Roles of CaMKKβ, AMPK and PI3K in the Endothelium

It has been recently demonstrated that the CaMKKβ-AMPK pathway phosphorylates eNOS at Ser1179 (human and mice eNOS Ser1177) via the PI3K-Akt pathway in vitro.[27] In order to examine the contribution of each component, we used STO-609 (a CaMKKβ inhibitor), compound C (an AMPK inhibitor) and wortmannin (a PI3K inhibitor). Importantly, STO-609, but not compound C or wortmannin, inhibited EDHF-mediated relaxations and hyperpolarizations to ACh only in mesenteric arteries, suggesting that CaMKKβ, but not AMPK or PI3K, plays an important role in the enhanced EDHF-mediated responses in mesenteric arteries. Although STO-609 had no effects on EDHF-mediated relaxations or hyperpolarizations in the aorta, it tended to inhibit eNOS phosphorylation at Ser1177 in the aorta. Thus, CaMKKβ might be involved in the functional activation of eNOS in mouse mesenteric arteries.

Role of Caveolin-1 in the Endothelium

In caveolin-$1^{-/-}$ mice, NO-mediated relaxations were enhanced in both the aorta and mesenteric arteries, whereas EDHF-mediated relaxations and hyperpolarizations were inhibited in mesenteric arteries. The present findings are consistent with those in recent reports.[38,39] These results suggest that functional suppression of eNOS by caveolin-1 is one of the key mechanisms for the enhanced EDHF-mediated responses in microvessels. We have previously demonstrated that eNOS-derived superoxide anions are the precursor of EDHF/H_2O_2 under physiological conditions.[16,17,24] In general, superoxide anions are produced from O_2^- and electrons in the reductase domain of eNOS.[40] In order to synthesize NO, eNOS requires an electron transfer from the reductase domain to the oxidase domain.[40] The caveolin-1-binding site is located in the eNOS oxygenase domain. However, it was reported that caveolin-1 interacts with the reductase domain and antagonizes CaM binding, thus compromising electron transfer from the reductase domain to the heme (in the oxygenase domain) and inhibiting NO synthesis.[41] It is conceivable that caveolin-1 inhibits the electron transfer, resulting in the enhanced production of EDHF/H_2O_2 from the reductase domains of eNOS (**Figure 8**). Further studies are needed to address this point.

Role of PKG1α in VSMC

PKG1α mediates H_2O_2-induced relaxation of VSMC, in which phosphorylation of BK_{Ca} channels is involved.[30] In the present study, Rp-8-Br-cGMPS, a PKG1α inhibitor, significantly inhibited VSMC relaxations and hyperpolarizations in response to exogenous H_2O_2 in mesenteric arteries but not in the aorta, suggesting that a PKG1α-mediated mechanism is involved in the enhanced EDHF-mediated responses in mesenteric arteries. It has recently been demonstrated that knock-in mice expressing only a C42S 'redox-dead' version of PKGI-α exhibit hypertension, suggesting the importance of EDHF/H_2O_2.[35] In those mice, SNP and NS-1619 caused a comparable extent of relaxation as in WT mice, suggesting that VSMC functions themselves are preserved.[35]

Study Limitations

Several limitations should be mentioned for the present study. First, although we carefully examined the endothelial and VSMC functions separately in isolated blood vessels, the present findings on the mechanisms for endothelial functions should be further confirmed in cultured endothelial cells from the aorta and mesenteric arteries in vitro. Second, the present findings in vitro remain to be confirmed in vivo in more detail. Third, it remained to be elucidated at which level of the conduit-resistance artery system such distinct transition of endothelial functions, from the NO-generating system in the conduit artery to the EDHF/H_2O_2-generating system in resistance arteries, occurs. Fourth, although we examined the role of PKG1α in VSMC, other possible mechanisms remain to be examined in future studies. Finally, it remains to be determined whether reactive oxygen scavenging enzymes, such as catalase and glutathione peroxidase, are involved in the mechanisms for the enhanced EDHF-mediated responses in microvessels.

Clinical Implications

In addition to NO-mediated responses, EDHF-mediated responses are substantially involved in the important cardiovascular regulatory mechanisms, including coronary autoregulation,[19] cardiovascular protection against myocardial ischemia/reperfusion injury[20] and metabolic coronary dilatation.[26] Furthermore, EDHF-mediated responses are relatively resistant to atherosclerotic injury, functioning as a back-up system for NO-mediated responses.[5,8] In the present study, we were able to elucidate the mechanisms for the enhanced EDHF-mediated responses in microvessels, including CAMKKβ and caveolin-1 (both in the endothelium) and PKG1α (in VSMC). Because EDHF-mediated responses are relatively resistant to atherosclerosis and thus are considered as a back-up system for NO-mediated responses,[5,8] the present findings might provide clues to develop a novel strategy for cardiovascular protection in humans.

Acknowledgments

We thank F. Natsui, F. Morikawa, N. Yamaki, A. Saito, C. Miyamoto and T. Hiroi for their excellent technical assistance.

This work was supported, in part, by the Grant-in-Aid for Scientific Research on Innovative Areas (Signaling Functions of Reactive Oxygen Species), the Grant-in-Aid for Tohoku University Global COE for Conquest of Signal Transduction Diseases with Network Medicine, and the Grants-in-Aid for Scientific Research, all of which are from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

Disclosures

None.

References

1. Feletou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: Where are we now? *Arterioscler Thromb Vasc Biol* 2006; **26:** 1215–1225.
2. Shimokawa H. Primary endothelial dysfunction: Atherosclerosis. *J Mol Cell Cardiol* 1999; **31:** 23–37.
3. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: Bringing the concepts together. *Trends Pharmacol Sci* 2002; **23:** 374–380.
4. Shimokawa H, Morikawa K. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J Mol Cell Cardiol* 2005; **39:** 725–732.
5. Shimokawa H. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pflugers Arch* 2010; **459:** 915–922.
6. Vanhoutte PM. Endothelial dysfunction: The first step toward coronary arteriosclerosis. *Circ J* 2009; **73:** 595–601.
7. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, 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.
8. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest* 1997; **100:** 2793–2799.
9. Rosolowsky M, Campbell WB. Role of PGI_2 and epoxyeicosatrienoic acids in relaxation of bovine coronary arteries to arachidonic acid. *Am J Physiol* 1993; **264:** H327–H335.
10. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 1999; **401:** 493–497.
11. Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* 1998; **396:** 269–272.
12. Beny JL, Schaad O. An evaluation of potassium ions as endothelium-derived hyperpolarizing factor in porcine coronary arteries. *Br J Pharmacol* 2000; **131:** 965–973.
13. Taylor HJ, Chaytor AT, Evans WH, Griffith TM. Inhibition of the gap junctional component of endothelium-dependent relaxations in rabbit iliac artery by 18-α glycyrrhetinic acid. *Br J Pharmacol* 1998; **125:** 1–3.
14. Yamamoto Y, Fukuta H, Nakahira Y, Suzuki H. Blockade by 18β-glycyrrhetinic acid of intercellular electrical coupling in guinea-pig arterioles. *J Physiol* 1998; **511** (Pt 2)**:** 501–508.
15. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 2000; **106:** 1521–1530.
16. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem Biophys Res Commun* 2002; **290:** 909–913.
17. Matoba T, Shimokawa H, Morikawa K, Kubota H, Kunihiro I, Urakami-Harasawa L, 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.
18. Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, Gutterman DD. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ Res* 2003; **92:** e31–e40.
19. Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Goto M, et al. Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation* 2003; **107:** 1040–1045.
20. Yada T, Shimokawa H, Hiramatsu O, Haruna Y, Morita Y, Kashihara N, et al. Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo. *Am J Physiol* 2006; **291:** H1138–H1146.
21. Lacza Z, Puskar M, Kis B, Perciaccante JV, Miller AW, Busija DW. Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am J Physiol* 2002; **283:** H406–H411.
22. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H, et al. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med* 2008; **205:** 2053–2063.
23. Takaki A, Morikawa K, Murayama Y, Yamagishi H, Hosoya M, Ohashi J, et al. Roles of endothelial oxidases in endothelium-derived hyperpolarizing factor responses in mice. *J Cardiovasc Pharmacol* 2008; **52:** 510–517.
24. Morikawa K, Shimokawa H, Matoba T, Kubota H, Akaike T, Talukder MA, et al. Pivotal role of Cu, Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest* 2003; **112:** 1871–1879.
25. Morikawa K, Fujiki T, Matoba T, Kubota H, Hatanaka M, Takahashi S, et al. Important role of superoxide dismutase in EDHF-mediated responses of human mesenteric arteries. *J Cardiovasc Pharmacol* 2004; **44:** 552–556.
26. Yada T, Shimokawa H, Hiramatsu O, Shinozaki Y, Mori H, Goto M, 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.
27. Levine YC, Li GK, Michel T. Agonist-modulated regulation of AMP-activated protein kinase (AMPK) in endothelial cells: Evidence for an AMPK→Rac1→Akt→endothelial nitric-oxide synthase pathway. *J Biol Chem* 2007; **282:** 20351–20364.
28. Li X, Han Y, Pang W, Li C, Xie X, Shyy JY, et al. AMP-activated protein kinase promotes the differentiation of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 2008; **28:** 1789–1795.
29. Potenza MA, Marasciulo FL, Chieppa DM, Brigiani GS, Formoso G, Quon MJ, et al. Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance between NO and ET-1 production. *Am J Physiol* 2005; **289:** H813–H822.
30. Burgoyne JR, Madhani M, Cuello F, Charles RL, Brennan JP, Schroder E, et al. Cysteine redox sensor in PKG1α enables oxidant-induced activation. *Science* 2007; **317:** 1393–1397.
31. Hosoya M, Ohashi J, Sawada A, Takaki A, Shimokawa H. Combination therapy with olmesartan and azelnidipine improves EDHF-mediated responses in diabetic apolipoprotein E-deficient mice. *Circ J* 2010; **74:** 798–806.
32. Gao JY, Yasuda S, Tsuburaya R, Ito Y, Shiroto T, Hao K, et al. Long-term treatment with eicosapentaenoic acid ameliorates myocardial ischemia-reperfusion injury in pigs in vivo: Involvement of Rho-kinase pathway inhibition. *Circ J* 2011; **75:** 1843–1851.
33. Fleming I. Molecular mechanisms underlying the activation of eNOS. *Pflugers Arch* 2010; **459:** 793–806.
34. Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji I, Kobayashi R. STO-609, a specific inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase kinase. *J Biol Chem* 2002; **277:** 15813–15818.
35. 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.
36. Hsu HH, Duning K, Meyer HH, Stolting M, Weide T, Kreusser S, et al. Hypertension in mice lacking the CXCR3 chemokine receptor.

Am J Physiol 2009; **296:** R780–R789.
37. Wang X, Lau F, Li L, Yoshikawa A, van Breemen C. Acetylcholine-sensitive intracellular Ca^{2+} store in fresh endothelial cells and evidence for ryanodine receptors. *Circ Res* 1995; **77:** 37–42.
38. Saliez J, Bouzin C, Rath G, Ghisdal P, Desjardins F, Rezzani R, et al. Role of caveolar compartmentation in endothelium-derived hyperpolarizing factor-mediated relaxation: Ca^{2+} signals and gap junction function are regulated by caveolin in endothelial cells. *Circulation* 2008; **117:** 1065–1074.
39. Pojoga LH, Yao TM, Sinha S, Ross RL, Lin JC, Raffetto JD, et al. Effect of dietary sodium on vasoconstriction and eNOS-mediated vascular relaxation in caveolin-1-deficient mice. *Am J Physiol* 2008; **294:** H1258–H1265.
40. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *J Biol Chem* 2001; **276:** 14533–14536.
41. Ghosh S, Gachhui R, Crooks C, Wu C, Lisanti MP, Stuehr DJ. Interaction between caveolin-1 and the reductase domain of endothelial nitric-oxide synthase: Consequences for catalysis. *J Biol Chem* 1998; **273:** 22267–22271.

Supplementary Files

Supplementary File 1

Figure S1. Effects of STO-609 on VSMC responses.

Figure S2. Effects of compound C on VSMC responses.

Figure S3. Effects of PI3K on VSMC responses.

Figure S4. Endothelium-independent relaxations in Cav-$1^{-/-}$ mice.

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-12-0197