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RESEARCH ARTICLE | *Vascular Biology and Microcirculation*

Important roles of endothelial caveolin-1 in endothelium-dependent hyperpolarization and ischemic angiogenesis in mice

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Ito A, Shiroto T, Godo S, Saito H, Tanaka S, Ikumi Y, Kajitani S, Satoh K, Shimokawa H. Important roles of endothelial caveolin-1 in endothelium-dependent hyperpolarization and ischemic angiogenesis in mice. *Am J Physiol Heart Circ Physiol* 316: H900–H910, 2019. First published February 1, 2019; doi:10.1152/ajpheart.00589.2018.—Although increased levels of reactive oxygen species (ROS) are involved in the pathogenesis of cardiovascular diseases, the importance of physiological ROS has also been emerging. We have previously demonstrated that endothelium-derived H_2O_2 is an endothelium-dependent hyperpolarization (EDH) factor and that loss of endothelial caveolin-1 reduces EDH/H_2O_2 in the microcirculation. Caveolin-1 (Cav-1) is a scaffolding/regulatory protein that interacts with diverse signaling pathways, including angiogenesis. However, it remains unclear whether endothelial Cav-1 plays a role in ischemic angiogenesis by modulating EDH/H_2O_2. In the present study, we thus addressed this issue in a mouse model of hindlimb ischemia using male endothelium-specific Cav-1 (eCav-1) knockout (KO) mice. In isometric tension experiments with femoral arteries from eCav-1-KO mice, reduced EDH-mediated relaxations to acetylcholine and desensitization of sodium nitroprusside-mediated endothelium-independent relaxations were noted (n = 4~6). An ex vivo aortic ring assay also showed that the extent of microvessel sprouting was significantly reduced in eCav-1-KO mice compared with wild-type (WT) littermates (n = 12 each). Blood flow recovery at 4 wk assessed with a laser speckle flowmeter after femoral artery ligation was significantly impaired in eCav-1-KO mice compared with WT littermates (n = 10 each) and was associated with reduced capillary density and muscle fibrosis in the legs (n = 6 each). Importantly, posttranslational protein modifications by reactive nitrogen species and ROS, as evaluated by thiol glutathione adducts and nitrotyrosine, respectively, were both increased in eCav-1-KO mice (n = 6~7 each). These results indicate that endothelial Cav-1 plays an important role in EDH-mediated vasodilatation and ischemic angiogenesis through posttranslational protein modifications by nitrooxidative stress in mice in vivo.

NEW & NOTEWORTHY Although increased levels of reactive oxygen species (ROS) are involved in the pathogenesis of cardiovascular diseases, the importance of physiological ROS has also been emerging. The present study provides a line of novel evidence that endothelial caveolin-1 plays important roles in endothelium-dependent hyperpolarization and ischemic angiogenesis in hindlimb ischemia in mice through posttranslational protein modifications by reactive nitrogen species and ROS in mice in vivo.

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angiogenesis; endothelium-dependent hyperpolarization; reactive oxygen species

INTRODUCTION

The endothelium plays important roles in modulating vascular tone by synthesizing and releasing several endothelium-derived relaxing factors (EDRFs), including vasodilator prostaglandins (PGs), nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors (33). EDH factors cause hyperpolarization and relaxation of underlying vascular smooth muscle cells (VSMCs). We have previously demonstrated that the contribution of EDRFs to endothelium-dependent vasodilatation markedly varies depending on vessel size; NO predominantly regulates the tone of large conduit vessels, while the importance of EDH increases as vessel size decreases (37). The nature of EDH factors varies depending on species and vascular beds examined, and several candidates have been proposed as the nature of EDH factors, including epoxyeicosatrienoic acids (metabolites of the arachidonic *P*-450 epoxygenase pathway) (5), electrical communication through gap junctions (13), K^+ (10), and H_2S (42). We have originally identified that endothelium-derived H_2O_2 is an EDH factor in mice (20) and human mesenteric arteries (18) and porcine coronary microvessels (19). Indeed, endothelium-derived H_2O_2 oxidizes and activates PKG-Iα in VSMCs, causing hyperpolarization and subsequent VSMC relaxations (27). We also have demonstrated that endothelial NO synthase (eNOS) is a major source of EDH factors (41), where Cu,Zn-superoxide dismutase catalyzes eNOS-derived superoxide anions to H_2O_2 (22). Thus, eNOS plays diverse roles depending on vessel size, as a NO-generating system in conduit arteries and an EDH-mediated system in resistance arteries (33, 35). Although NO and EDH are well balanced in a vessel-size-dependent manner under physiological conditions, the balance could be impaired under various pathological conditions, such as aging, dyslipidemia, hypertension, and diabetes mellitus, all of which are known risk factors of cardiovascular diseases (CVD) (43).

Increased levels of reactive oxygen species (ROS) are known to be involved in the pathogenesis of CVD; the importance of physiological ROS has also been emerging. The balance between oxidants and antioxidants regulates intracellular redox state, and CVD often results from the imbalance in levels of intracellular ROS (31). Indeed, in the pathological redox state, excessive ROS (oxidative stress) are produced by external stimuli, such as mechanical stretch, pressure, shear

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stress, and hypoxia (35), leading to endothelial dysfunction and VSMC proliferation (34). On the other hand, physiological levels of ROS regulate cell function, proliferation, normal levels of cell death, and vasodilatation (31). In addition, reactive oxygen and nitrogen species (RONS) are increased in ischemic tissues and necessary for revascularization (6). Since low-molecular-weight thiol glutathione (GSH) reacts with RONS and protein cysteines during ischemia with resultant production of proangiogenic GSH adducts, mice with genetic overexpression or deletion of glutaredoxin-1, which selectively removes GSH adducts, have impaired or improved revascularization in ischemic hindlimb models, respectively (23, 46). However, it remains to be elucidated whether physiological concentrations of ROS modulate ischemic angiogenesis.

Caveolin-1 (Cav-1) is a scaffolding/regulatory protein localized in caveolae, flask-shaped invaginations of the plasma membrane, and is particularly abundant in endothelial cells (ECs) and adipocytes (39). In the caveolae, Cav-1 interacts with various signaling molecules, including G protein-coupled receptors, tyrosine kinases, and GTPases, and inhibits NO production by binding to eNOS (4, 28). Although systemic Cav-1 knockout (KO) mice show enhanced NO-dependent vascular responses (9, 28), they rather develop cardiomyopathy and pulmonary hypertension due to persistent eNOS activation (48). With regard to ischemic angiogenesis, a previous report has shown that systemic Cav-1-KO mice failed to recover a functional vasculature in hindlimb ischemia-reperfusion (38), whereas mice with endothelium-specific overexpression of Cav-1 had impaired angiogenesis in the same model (3). These data suggest a complex role of Cav-1 in the regulation of angiogenesis.

We have previously demonstrated that endothelial Cav-1 is substantially involved in mechanisms for enhanced EDH-mediated responses in the microcirculation in mice (12, 25). Recently, we newly generated endothelium-specific Cav-1 (eCav-1)-KO mice to reduce EDH, demonstrating that endothelial Cav-1 is involved in the protective role of EDH against nitrative stress by excessive NO (29). However, the importance of EDH and endothelial Cav-1 in ischemic angiogenesis remains to be examined. In the present study, we thus explored the importance of EDH and endothelial Cav-1 in a mouse model of hindlimb ischemia using eCav-1-KO mice.

METHODS

Animals. The present study was reviewed and approved by the Committee on Ethics of Animal Experiments of Tohoku University (nos. 2016MdA-269 and 2018MdA-001), which was granted by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experiments were conducted in ~8- to 16-wk-old male mice, with mice aged 8~12 wk used for the aortic ring assay and mice aged 12~16 wk for other experiments. All animals were maintained in the Laboratory of Animal Experiments in Tohoku University and were cared for in accordance with the rules and regulations configured by the committee, fed standard rodent chow, and maintained on a 12:12-h light-dark cycle.

Generation of eCav-1-KO mice. We have recently developed eCav-1-KO mice as previously described (29). Briefly, Cav-1-floxed (Cav-1flox/flox) mice, in which Cav-1 exon 3 is flanked by two loxP sites on the C57BL6 background (Unitech, Kashiwa, Japan), were crossbred with Tie2-Cre mice (Jackson Laboratory, Bar Harbor, ME). Cav-1flox/flox Tie2-Cre-positive mice were used as eCav-1-KO mice, and Cav-1flox/flox Tie2-Cre negative littermates were used as control mice in the present study. The genotype of the mice was confirmed by PCR using primers specific for the targeted gene (5′-TATTCTCCTTGCTCTAATGTCACCT-3′ and 5′-ACAGTGAGGGTCTTTGAAGATGTTA-3′) and the Tie2-Cre transgene (5′-GCGGTCTGGCAGTAAAAACTATC-3′ and 5′-GTGAAACAGCATTGCTGTCACTT-3′) (29).

Organ chamber experiments. We measured isometric tensions of femoral arteries as previously described (11). After mice were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), femoral 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% O_2-5% CO_2 at 37°C and were then stretched to optimal resting tensions. After a 60-min equilibration period, rings were challenged with KCl (60 mmol/l) to confirm their viability; rings that were able to generate >1 mN of force were allowed for the following isometric tension recordings. After washout and a 30-min recovery period, the rings were precontracted with phenylephrine (10^{-6} mol/l) to examine the relaxation responses to acetylcholine (ACh; 10^{-10}–10^{-5} mol/l). The contributions of vasodilator PGs, NO, and EDH to ACh-induced endothelium-dependent relaxations were determined by the inhibitory effect of indomethacin (Indo; cyclooxygenase inhibitor, 10^{-5} mol/l), N^{ω}-nitro-L-arginine (L-NNA; NOS inhibitor, 10^{-4} mol/l), and a combination of charybdotoxin [CTx; intermediate- and large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel blocker, 10^{-7} mol/l] plus apamin (small-conductance K_{Ca} channel blocker, 10^{-6} mol/l), respectively (11). The concentration of Indo was sufficient to inhibit the production of vasodilator PGs, as evidenced by the fact that additional application of tranylcypromine (100 mM), an inhibitor of prostacyclin synthase, had no further inhibitory effect on Indo-resistant relaxation, and the concentration of L-NNA was sufficient to inhibit endothelial NO production, as evidenced by the fact that additional application of carboxy-2-(4-carboxyphenyl)-4,4,5,5,tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) (5 mM), a potent scavenger of NO, had no further inhibitory effect on L-NNA-resistant relaxation (20, 22). All inhibitors were applied to organ chambers 30 min before precontraction with phenylephrine. To assess endothelium-independent relaxations, the NO donor sodium nitroprusside (SNP; 10^{-10}–10^{-5} mol/l) and the K^+ channel opener NS-1619 (10^{-8}–10^{-4} mol/l) were used in endothelium-denuded arteries. Vascular responses to exogenous H_2O_2 (10^{-8}–10^{-3} mol/l) were examined in the presence of Indo (10^{-5} mol/l) and L-NNA (10^{-4} mol/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. Responses were monitored by a computer-based analysis system in LabChart 7.0 software (PowerLab 8/30 computer system, AD Instruments, Colorado Springs, CO) (12).

Aortic ring assay. The aortic ring assay was performed as previously described (24). Briefly, the descending thoracic aorta was isolated, and 1-mm-long aortic rings were embedded in growth factor-reduced Matrigel supplemented with 20 U/ml heparin. The aortic rings were then cultured in endothelial basal medium (BD Biosciences, San Jose, CA) (2) supplemented with or without 30 ng/ml human recombinant VEGF (PreproTech, Rocky Hill, NJ) and/or 1% penicillin-streptomycin at 37°C. Microvessel area was quantified by WinROOF 2015 software (Mitani, Tokyo, Japan) under a blinded manner.

Hindlimb ischemia model. Animals were anesthetized with 1–3% isoflurane at a flow rate of 300–400 ml/min. After being anesthetized, mice were placed on a 37°C heating pad to avoid hypothermia, and the rectal temperature was monitored to check that it remained within the range of 36.5–37.5°C. Hindlimb ischemia was made by left femoral artery ligation as previously described (24). A single dose of bu-

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prenorphine (0.1 mg/kg) was administered subcutaneously to control ischemic pain.

Measurement of blood flow. We measured the plantar blood flow ratio in the ischemic (left) to nonischemic (right) limb using a laser speckle blood flowmeter (Omega Zone, Omegawave, Tokyo, Japan) as previously described (17). The flow was continuously measured with high-speed average mode (15 images/s) using a charge-coupled device camera in the prone position with a sticky pad to show the plantar aspect on a heating pad under inhalation anesthesia with 1.0% isoflurane, and body temperature was thermostatically maintained at 36.5–37.5°C via a rectal temperature probe. Blood flow was measured on *day 0* (before and after ligation surgery) and *days 3*, *7*, *14*, *21*, and *28*. The average blood flow ratio of 10 measurements was calculated on the basis of colored histogram pixels.

Histological analysis. Under deep anesthesia with isoflurane, the mouse was perfused with 37–39°C heparin solution [Dulbecco's PBS (DPBS) and heparin (10 U/ml)] followed by vasodilation solution for morphometry containing adenosine (100 μmol/l), SNP (10 μmol/l), and BSA [0.05% (wt/vol)] by directly dissolving the reagents with DPBS with Ca^{2+} and Mg^{2+} and was fixed with 4% paraformaldehyde phosphate buffer solution via the left ventricle of the heart. Next, gastrocnemius muscles were harvested, immersed in 4% buffered paraformaldehyde for 24 h and 90% ethanol for another 24 h, embedded in paraffin, and cut into 3-μm-thick sections. Serial sections were stained with hematoxylin and eosin to determine the cross-sectional area (CSA) of myocytes, with Masson's trichrome for the interstitial or perivascular fibrosis area, and immunostained with anti-CD31 for capillary density. CSA, fibrosis area, and capillary density were calculated with BZ-X analyzer.exe version 1.3.1.1. (Keyence, Osaka, Japan). All analyzing procedures were performed in a blinded manner.

Western blot analysis. We performed Western blot analysis as previously described (29). Gastrocnemius muscles were isolated and snap frozen. Frozen muscles were homogenized with both tissue protein extract reagent (T-PER; ThermoFisher Scientific, Rockford, MA) and protease inhibitor cocktail (Sigma-Aldrich). After centrifugation, supernatants of the lysates were loaded on SDS-PAGE gels and transferred to PVDF membranes (GE Healthcare, Fairfield County, CT). For detection of GSH adducts, proteins were analyzed in the nonreducing condition. Membranes were blocked for 1 h at room temperature. Regions containing proteins were visualized by the enhanced chemiluminescence system (ECL Prime Western Blotting Detection Reagent, GE Healthcare). Densitometric analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD). If necessary, blots were stripped with Western blot stripping solution (Nacalai Tesque, Kyoto, Japan) and reprobed. Finally, equal loading was determined by protein concentration measured with the BCA assay (BCA Protein Assay Kit, ThermoFisher Scientific) or probing for GAPDH.

Measurement of oxidative and nitrative stress. To assess oxidative and nitrative stress in tissue, we measured the ratio of oxidized to reduced glutathione (GSSG/GSH ratio) and 3-nitrotyrosine (3-NT) using commercially available ELISA kits following the manufacturer's instructions, respectively.

Materials. CTx was purchased from Peptide Institute (Osaka, Japan), SNP from Maruishi Seiyaku (Osaka, Japan), and all other drugs for organ chamber experiment from Sigma-Aldrich. Indo was dissolved in 10 mmol/l Na_2CO_3, NS-1619 in DMSO, and the others in distillated water. The ionic composition of KHB was as follows (in mmol/l): 144 Na^+, 5.9 K^+, 1.2 Mg^{2+}, 2.5 Ca^{2+}, 1.2 $H_2PO_4^{3-}$, 24 HCO_3^-, 129.7 Cl^-, and 5.5 glucose. DPBS with Ca^{2+} and Mg^{2+} was purchased from BioWhittaker, 4% paraformaldehyde phosphate buffer solution from Wako Pure Chemical Industries (Osaka, Japan), and Opti-MEM + GlutaMAX-I from GIBCO (ThermoFisher Scientific). The primary antibodies used were as follows: 3-NT (667 ng/ml, Abcam, Cambridge, UK), Akt (34.0 ng/ml, Cell Signaling Technology, Danvers, MA), phosphorylated (p-)Ser^{473} Akt (180 ng/ml, Cell Signaling Technology), CD31 (20 μg/ml, Abcam), eNOS (250 ng/ml, BD Transduction Laboratories), p-Ser^{1177} eNOS (250 ng/ml, BD Transduction Laboratories), GAPDH (4.2 ng/ml, Cell Signaling Technology), glutathione (1 μg/ml, Abcam), and VEGF receptor-2 (VEGFR-2; 100 ng/ml, Cell Signaling Technology). A 3-NT ELISA kit was purchased from Abcam. The glutathione assay kit was purchased from Cayman Chemical.

Statistical analysis. All results are expressed as means ± SE. All parameters were evaluated with an two-tailed unpaired Student's *t*-test or compared by two-way ANOVA followed by a Tukey's test for multiple comparisons. Dose-response curves between groups were compared by two-way ANOVA followed by Tukey's multiple-comparisons tests. Statistical analysis was performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA). Results were considered to be statistically significant at $P < 0.05$.

RESULTS

Endothelium-dependent relaxations in femoral arteries. To explore the contribution of EDRF in femoral arteries in mice, we performed organ chamber experiments to measure isometric tensions. Outer and inner diameters of femoral arteries were comparable between eCav-1-KO and control mouse groups (outer diameter: 360.0 ± 6.1 vs. 347.5 ± 38.4 μm and inner diameter: 250.0 ± 13.7 vs. 237.5 ± 7.9 μm, $n = 5$ each). Contraction responses to KCl (60 mmol/l) in isolated femoral arteries were comparable between eCav-1-KO and control mice (data not shown). Assessment of endothelium-dependent relaxations to ACh of femoral arteries showed that eCav-1-KO mice had slightly but significantly enhanced relaxation responses (Fig. 1, *A* and *B*). In the area under the curve analysis, EDH-mediated relaxations in femoral arteries were dominant in control mice but were significantly attenuated in eCav-1-KO mice, whereas NO-mediated relaxations were comparable between the two genotypes (Fig. 1, *C*−*E*). Vasodilator PG-mediated relaxations also tended to be enhanced in eCav-1-KO mice (Fig. 1, *D* and *E*).

Endothelium-independent relaxation in femoral arteries. We also performed organ chamber experiments to measure endothelium-independent relaxation of VSMCs of femoral arteries. Relaxations to the K^+ channel opener NS-1619 were comparable between the two genotypes (Fig. 2, *A* and *B*), but there was a rightward shift in the dose-response curve to the NO donor SNP in eCav-1-KO mice compared with control mice (Fig. 2, *C* and *D*). Relaxations to exogenous H_2O_2 were significantly attenuated in eCav-1-KO mice, although the difference seemed small in terms of EC_{50} (Fig. 2, *E* and *F*).

Impaired microvessel sprouting in the aorta of eCav-1-KO mice. Next, we examined the ex vivo aortic ring assay to explore the ability of angiogenesis in eCav-1-KO mice. In the presence of 30 ng/ml VEGF, isolated aortic rings from eCav-1-KO mice showed significantly less microvessel sprouting compared with control mice (Fig. 3, *A* and *B*), indicating that endothelial Cav-1 maintains the ability of the angiogenic response.

Impaired angiogenesis in eCav-1-KO mice after hindlimb ischemia. To examine whether endothelial Cav-1 contributes to ischemic angiogenesis in vivo, the extent of blood flow recovery was assessed in the ischemic hindlimb model. Immediately after femoral artery ligation, blood flow in the ischemic limb was equally decreased in both genotypes (Fig. 4, *A* and *B*).

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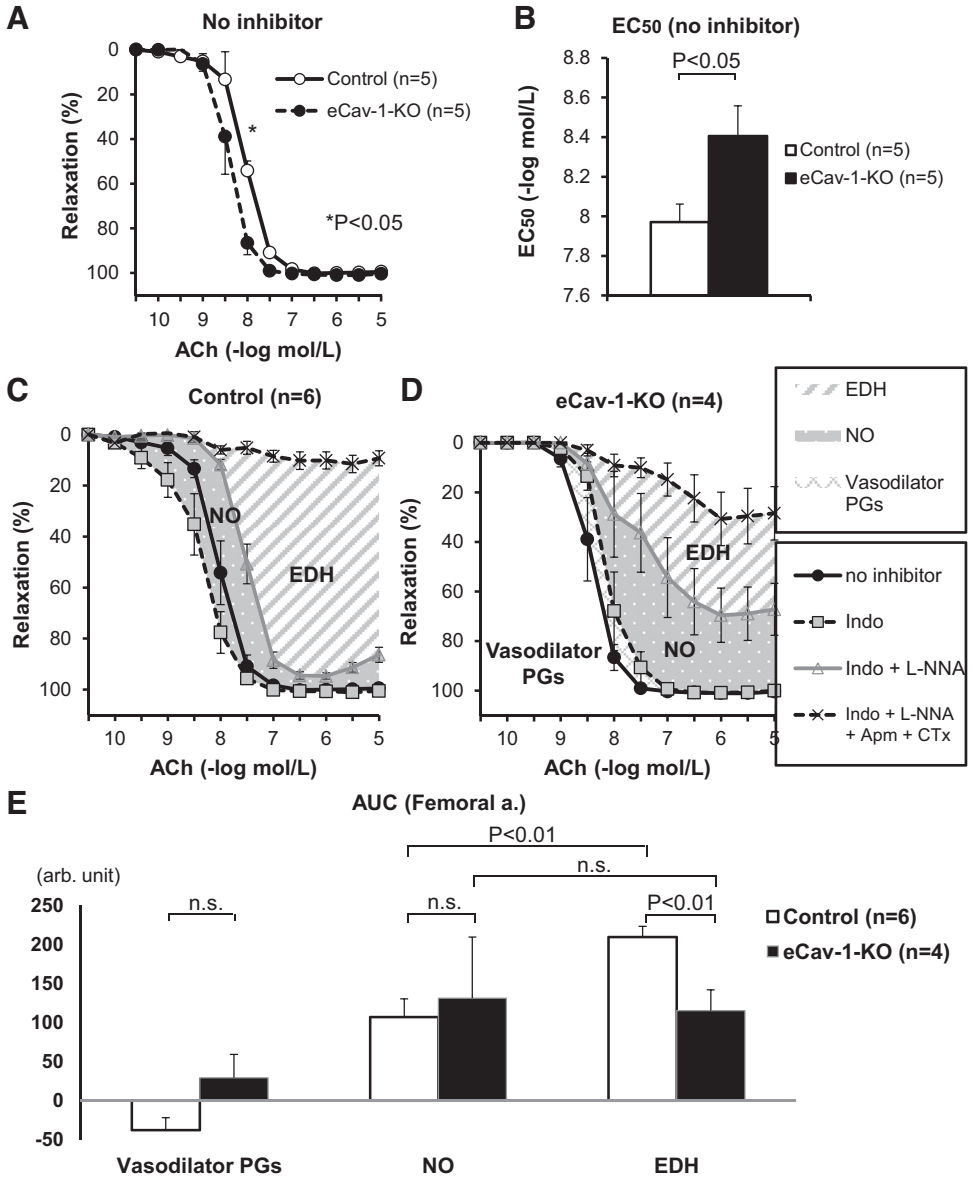

Fig. 1. Endothelium-dependent relaxations in femoral arteries of mice. *A*: endothelium-dependent relaxations to acetylcholine (ACh; $10^{-10}-10^{-5}$ mol/l) in control and endothelium-specific caveolin-1 (eCav-1) knockout (KO) mice without inhibitors (n = 5 mice/group). *B*: EC_{50} of endothelium-dependent relaxations to ACh in control and eCav-1-KO mice. *C* and *D*: ACh-induced endothelium-dependent relaxations in control (*C*) and eCav-1-KO (*D*) mice (n = 4~6). The contributions of vasodilator prostaglandins (vasodilator PGs), nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) were determined by the inhibitory effect of indomethacin (Indo; 10^{-5} mol/l), N^{ω}-nitro-L-arginine (L-NNA; 10^{-4} mol/l), and a combination of apamin (Apm; 10^{-6} mol/l) plus charybdotoxin (CTx; 10^{-7} mol/l), respectively. *E*: relative contributions of vasodilator PGs, NO, and EDH to endothelium-dependent relaxations among the two genotypes were compared with the area under the curve. Results are expressed as means ± SE. n.s., Not significant.

However, percent blood flow recovery in the ischemic limb at *day 28* was significantly decreased in eCav-1-KO mice compared with control mice (eCav-1-KO mice: 62.5 ± 3.8% vs. control mice: 72.0 ± 2.4%; Fig. 4, *A* and *B*). Histological analysis showed that capillary density in the ischemic limb was significantly decreased in eCav-1-KO mice (Fig. 4, *C* and *D*). Similarly, Masson's trichrome staining showed that the extent of fibrosis was also deceased in eCav-1-KO mice compared with control mice (Fig. 4, *E* and *F*).

Reduced VEGFR-2 causes impaired angiogenic signaling in eCav-1-KO mice. For further understanding of the underlying mechanism(s) of impaired ischemic angiogenesis in eCav-1-KO mice, we performed Western blot analysis of gastrocnemius muscle. In WT mice, the extent of VEGFR-2 protein expression in the ischemic limb was significantly increased compared with the nonischemic limb, whereas in eCav-1-KO mice it was significantly reduced compared with the control nonischemic limb, although the extent of VEGF expression in the ischemic limb was comparable between the two genotypes (Fig. 5, *A* and *B*). The extent of Akt phosphorylation at Ser^{473}, a well-known downstream target of VEGFR-2, was significantly reduced in eCav-1-KO mice, which was also the case for phosphorylations of Akt at Ser^{473} and eNOS at stimulatory Ser^{1177} (Fig. 5, *C*−*E*).

Assessment of oxidative and nitrative stress in hindlimb ischemia. To further explore the mechanisms of reduced VEGFR-2 expression and phosphorylations of Akt and eNOS in eCav-1-KO mice, we evaluated protein GSH adducts as a biomarker of RONS. Protein GSH adducts that promote angiogenesis tended to be increased in eCav-1-KO mice (Fig. 6, *A* and *C*). We also measured the GSSG/GSH ratio, a marker of oxidative stress, and found that ischemic muscle in eCav-1-KO mice had higher oxidative stress compared with control mice (Fig. 6*E*). We then evaluated nitrative stress in ischemic tissue by 3-NT, a biomarker of reactive nitrogen species (6), and found that 3-NT levels in ischemic muscle were significantly higher in eCav-1-KO mice compared with control mice (Fig. 6, *B*, *D*, and *F*).

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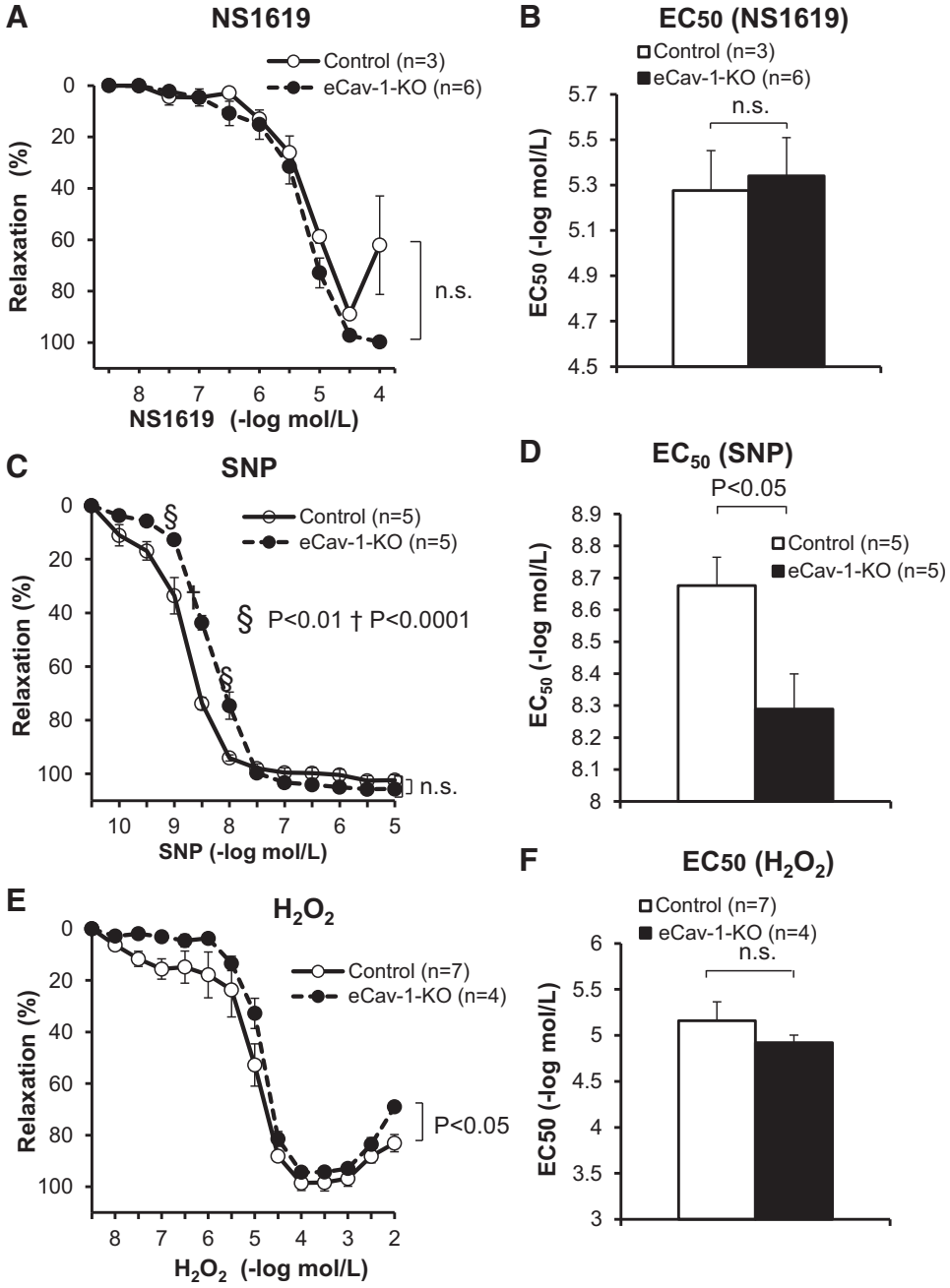

Fig. 2. Endothelium-independent relaxations in femoral arteries of mice. Endothelium-independent relaxations to NS-1619 (*A*), sodium nitroprusside (SNP; *C*), and H_2O_2 (*E*) as well as their EC_{50} values (*B*, *D*, and *F*) are shown. Rresponses to H_2O_2 were examined in the presence of indomethacin (10^{-5} mol/l) and N^{ω}-nitro-L-arginine (L-NNA; 10^{-4} mol/l). All results are expressed as means ± SE. §$P < 0.01$' †$P < 0.0001$. n.s., Not significant.

DISCUSSION

The novel findings of the present study with eCav-1-KO mice were as follows. First, EDH is the major EDRF in femoral arteries in mice. Second, endothelium-specific deletion of Cav-1 causes impaired ischemic angiogenesis. Third, despite enhanced expression of GSH adducts (*S*-glutathionylation), angiogenesis and muscle fibrosis after femoral artery ligation are reduced in eCav-1-KO mice. Fourth, *S*-nitrosylation in eCav-1-KO mice is significantly increased, especially in the hindlimb, indicating enhanced nitrative stress in those mice. To the best of our knowledge, this is the first study that has demonstrated that endothelial Cav-1 plays an important role in EDH-mediated vasodilatation and ischemic angiogenesis through posttranslational protein modifications by nitrooxidative stress in mice in vivo.

Endothelium-dependent and -independent relaxation in femoral arteries. We previously demonstrated that endothelium-derived H_2O_2 is an EDH factor (20) and that the importance of EDH/H_2O_2 increases as the vessel size decreases (37). We have previously demonstrated that ACh-induced relaxations are also enhanced in the aorta from eCav-1-KO mice (29). Although femoral arteries are recognized as conduit arteries in anatomy, the present study demonstrated that EDH-mediated relaxations were dominant in femoral arteries in WT mice, the diameters of which were relatively small (~350 μm). Thus, vascular function needs to be evaluated not only for anatomy

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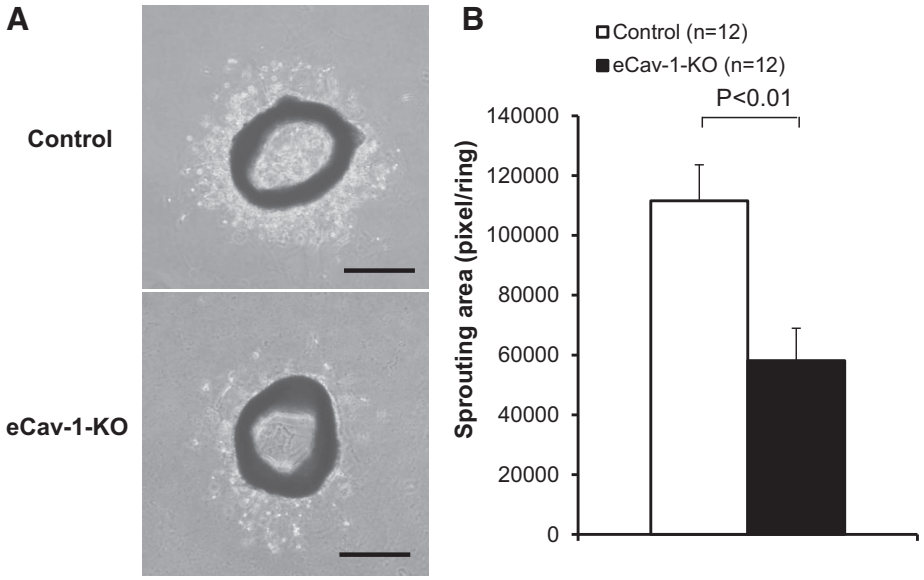

Fig. 3. Impaired microvessel sprouting in the aorta of endothelium-specific caveolin-1 (eCav-1) knockout (KO) mice. *A*: representative micrographs of sprouting microvessels from isolated aortic rings grown in the presence of 30 ng/ml VEGF. Scale bars = 500 μm. *B*: area of the sprouting microvessels from aortic rings in control and eCav-1-KO mice (n = 12 each). Results are expressed as means ± SE.

(e.g., conduit vs. resistant) but also for vessel size. Consistent with the present finding, Yu et al. (47) also reported similar endothelium-dependent relaxation curves to ACh with or without Indo plus L-NNA in femoral arteries of mice. We also found that EDH-mediated relaxations were significantly attenuated in femoral arteries from eCav-1-KO mice, indicating that endothelial Cav-1 is a key regulator of EDH-mediated relaxations in femoral and mesenteric arteries (29).

Interestingly, with regard to endothelium-independent relaxation, we observed that relaxations to NS-1619 were comparable between the two genotypes, whereas there was a rightward shift in the dose-response curve to SNP in eCav-1-KO mice compared with control mice, suggesting impaired NO sensitivity of VSMCs. Although the underlying mechanism remains to be elucidated, it has been previously reported that persistent eNOS activation secondary to the loss of Cav-1 causes exaggerated NO production with resultant inhibition of PKG due to nitration (49).

Impaired angiogenesis after hindlimb ischemia in eCav-1-KO mice. In the present study, we demonstrated impaired angiogenesis in eCav-1-KO mice in both the isolated aortic ring assay ex vivo and a hindlimb ischemia model in vivo. Sonveaux et al. (38) previously reported similar results of impaired angiogenesis in endothelial network formation in vitro and a hindlimb ischemia model in vivo in systemic Cav-1-KO mice. However, it remains to be examined whether endothelial Cav-1 itself directly modulates ischemic angiogenesis because Cav-1 also exists in VSMCs and other types of cells (26). In the present study, the extent of VEGF protein expression was comparable between the two genotypes, whereas that of VEGFR-2 expression was significantly reduced in eCav-1-KO mice. Consistently, phosphorylations of Akt at Ser^{473} and eNOS at Ser^{1177} were both reduced in those mice. VEGFR-2 is known to be localized to the caveolae, and its disruption by cholesterol depletion leads to inhibition of VEGF-induced ERK activation, suggesting that its localization to the caveolae is crucial for VEGF-mediated signaling (15). In addition, Sonveaux et al. (38) also examined the rescue model in which aortic ECs from systemic Cav-1-KO mice were transfected with caveolin plasmid, showing that those ECs restored VEGF-induced ERK activation. Thus, endthelial Cav-1 appears to be a key regulator of VEGF-mediated angiogenesis. On the other hand, Cav-1 is also known to modulate EC permeability by regulating eNOS activity (32). Interestingly, Bauer et al. (3) reported impaired microvascular permeability and angiogenesis in the hindlimb ischemia model in mice with endothelium-specific overexpression of Cav-1. Taken together, these results indicate that Cav-1 may not modulate ischemic angiogenesis by regulating EC permeability and that fine-tuned endothelial Cav-1 abundance is essential for proper ischemic angiogenesis.

In the present study, we also demonstrated that eCav-1-KO mice had enhanced endothelium-dependent relaxations but reduced blood flow after the induction of hindlimb ischemia. This discrepancy suggests that NO is unable to work as a vasodilator in ischemic and inflamed conditions where vascular Rho-kinase activity is upregulated (36). Indeed, Rho-kinase is an important functional inhibitor of eNOS in vivo (33, 36). Hatoum et al. (14) previously reported that inflammatory bowel disease is associated with microvascular dysfunction and that NO-mediated vasodilation was absent in human inflamed microvessels with inflammatory bowel disease, possibly resulting in reduced perfusion, poor wound healing, and sustained chronic inflammation. Interestingly, Aggarwal et al. (1) previously reported that nitration of Tyr^{247} inhibits PKG-1α activity by attenuating cGMP binding. Indeed, in the present study, the extent of nitrative stress in the ischemic limb was significantly higher in eCav-1-KO mice than in control mice. Thus, it is possible that nitrative stress causes subsequent PKG nitration and PKG inactivation, attenuating relaxations through the NO-cGMP-PKG pathway. It is possible that such ischemic and inflamed condition causes loss of NO-dependent vasodilation, and the residual vasodilators (vasodilator PGs and EDH) may compensate blood flow recovery. In line with this notion, we found that EDH-mediated relaxations were reduced in femoral arteries of eCav-1-KO mice. Taken together, EDH may work as a compensatory vasodilator in the

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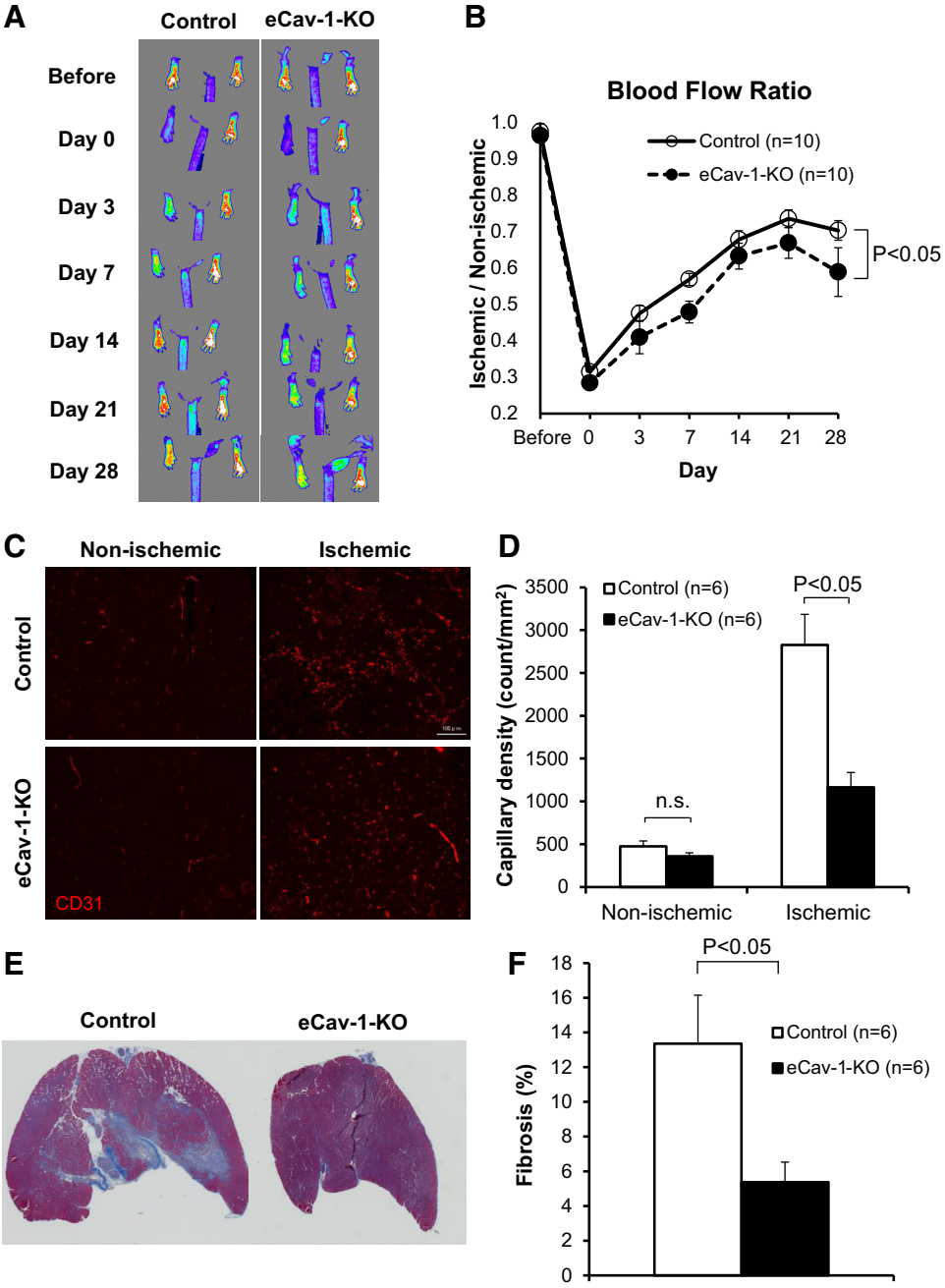

Fig. 4. Impaired angiogenic responses in ischemic limb of endothelium-specific caveolin-1 (eCav-1) knockout (KO) mice. *A*: representative images of laser speckle blood flow in control and eCav-1-KO mice. *B*: blood flow in the ischemic limb was measured before, immediately after, and on *days 3*, *7*, *14*, *21*, and *28* after ligation of the left femoral artery ($n = 10$ each). Results are expressed as a ratio of the left (ischemic) to right (nonischemic) limb perfusion. *C*: representative capillary staining in gastrocnemius muscle sections from control and eCav-1-KO mice at 4 wk after hindlimb ischemia. Original magnification: ×20. *D*: quantitative analysis of capillary density at 4 wk after ischemia ($n = 6$ each). Results are expressed as means ± SE. *E*: representative images of Masson's trichrome staining for fibrosis. *F*: quantitative analysis of fibrosis area at 4 wk after the ischemia ($n = 6$ each). Results are expressed as means ± SE. n.s., Not significant.

femoral and other resistance arteries, maintaining blood flow in hindlimb ischemia.

Oxidative and nitrative stress as the underlying mechanism in histology of eCav-1-KO mice. The present study revealed that angiogenesis and fibrosis after femoral artery ligation were significantly reduced in eCav-1-KO mice, whereas protein *S*-glutathionylation and *S*-nitrosylation were significantly enhanced. The importance of protein *S*-glutathionylation in angiogenesis has been recently reported, and the generation of GSH adducts is mainly mediated through three ways, including disulfide bond formation among proteins with sulfenic acid formation, thiol exchange between GSSG, and chemically unstable *S*-nitrosylation (45). This means that *S*-glutathionylation is generated with specific proteins, such as SERCA and NF-κB with physiological but not so severe RONS because excessive ROS cause derailment of reducible sulfenic formation and oxidized into biologically irreversible oxidative modifications (45). Thus, it is expected to note enhanced *S*-nitrosylation and *S*-glutathionylation in ischemic tissue, especially from eCav-1-KO mice, because there must be abundant or excessive NO. Mechanistically, it is well known that the NO-cGMP-PKG pathway has been implicated in ischemic angiogenesis (7). Consistent with our findings, it has been previously reported that chronic hypoxia increases tyrosine nitration of PKG in the right ventricle of systemic Cav-1-KO mice (8). Thus, it is conceivable that loss of endothelial Cav-1 induces PKG nitrosylation and decreases its activity, impairing ischemic angiogenesis, for which posttranslational modifica-

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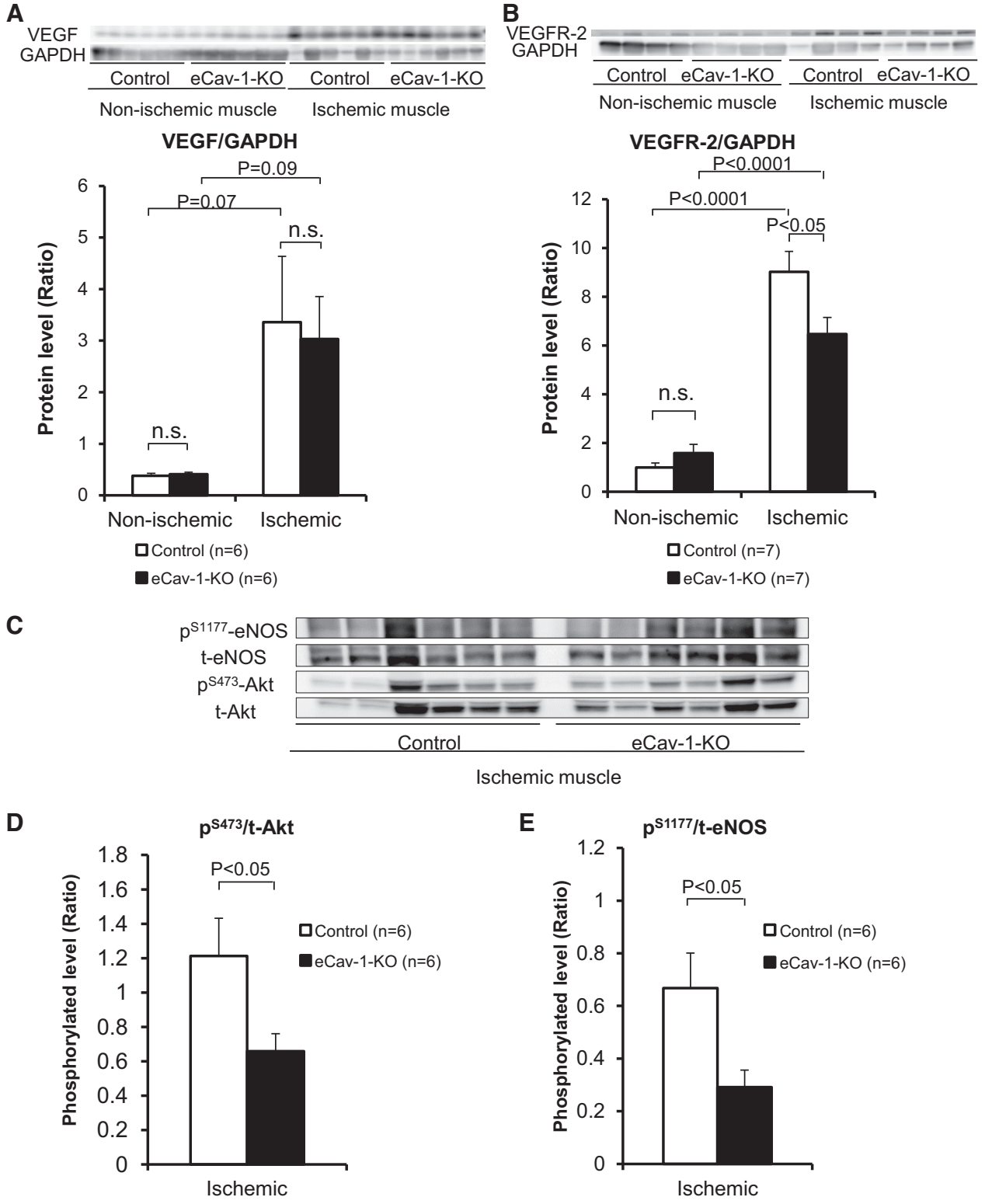

Fig. 5. Changes of angiogenic signaling molecules in endothelium-specific caveolin-1 (eCav-1) knockout (KO) mice. Western blot analysis was performed using ischemic gastrocnemius muscles. *A* and *B*: representative blot and quantitative densitometry analysis of VEGF (*A*; $n = 6$ each) and VEGF receptor-2 (VEFGR2; *B*; $n = 7$ each). Results are expressed as means ± SE. *C*: representative blots of Akt and endothelial nitric oxide synthase (eNOS). *D* and *E*: quantitative densitometry analysis of phosphorylated Akt at Ser^{473} (p^{S473}-Akt; *D*) ($n = 6$ each) and phosphorylated eNOS at Ser^{1177} (p^{S1177}-eNOS; *E*; $n = 6$ each). t-Akt, total Akt; t-eNOS, total eNOS. Results are expressed as means ± SE.

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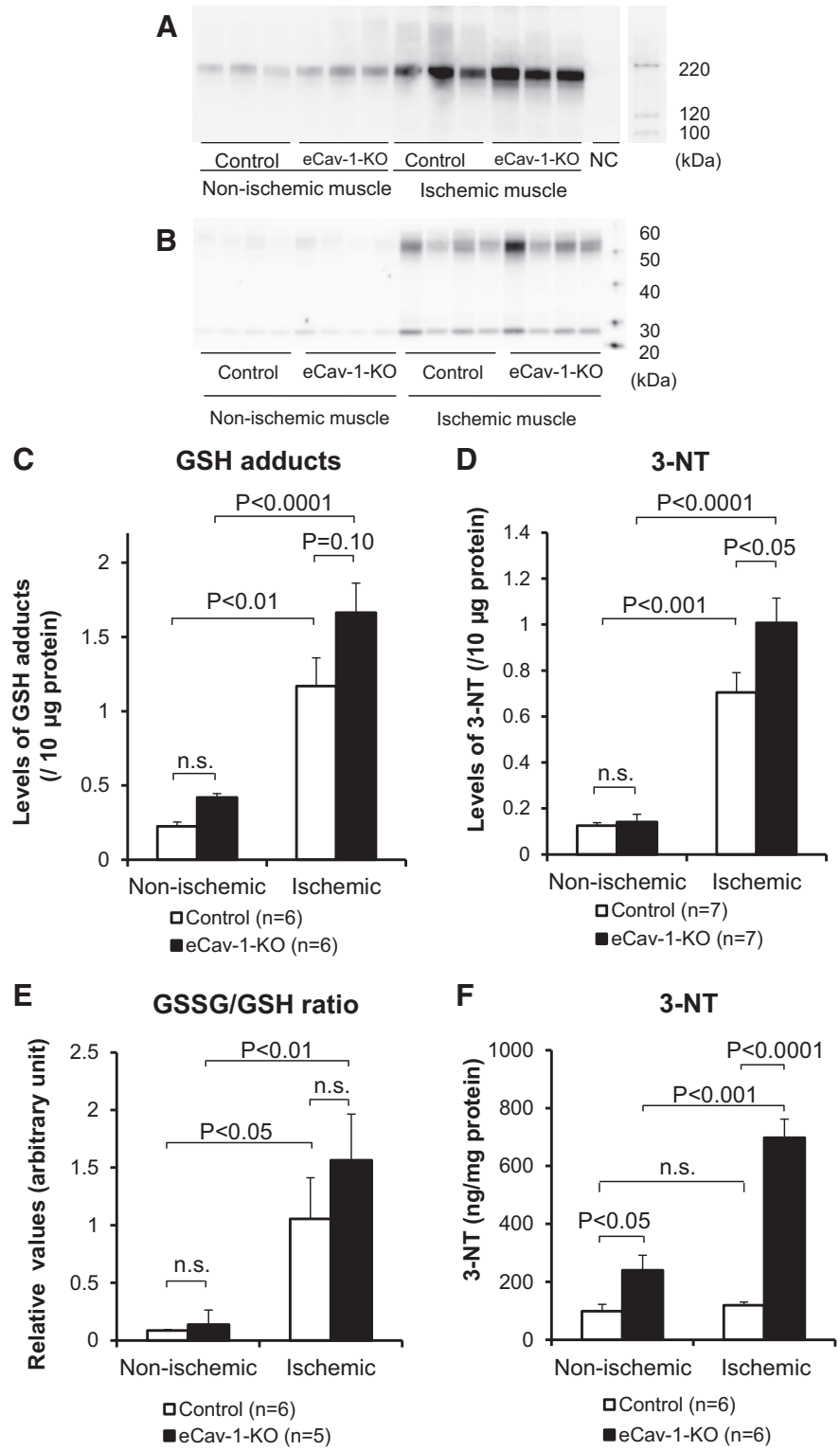

Fig. 6. Oxidative and nitrative stress of the hindlimb tissue after ischemia. *A* and *C*: levels of protein glutathione (GSH) adducts in ischemic gastrocnemius muscles of control and endothelium-specific caveolin-1 (eCav-1) knockout (KO) mice were detected by anti-GSH antibody. Representative blot (*A*) and densitometry analysis (*C*) are shown. *B* and *D*: levels of 3-nitrotyrosine (3-NT) in ischemic gastrocnemius muscles. Representative blot (*B*) and densitometry analysis (*D*) are shown. *E* and *F*: ratio of oxidized to reduced glutathione (GSSG/GSH ratio; *E*) and levels of 3-NT (*F*) in ischemic gastrocnemius muscles measured by ELISA. Results are expressed as means ± SE.

tion of *S*-nitrosylation by excessive NO may be involved. Furthermore, scavenging H_2O_2 by overexpression of catalase inhibits angiogenesis in hindlimb ischemia in vivo (44), which also indicates the important role of EDH/H_2O_2 in ischemic angiogenesis.

Study limitations. Several limitations should be mentioned for the present study. First, although we used Tie2 protein as a driver for EC expression in the present study, it is also expressed in hematopoietic lineages. Second, to address the role of EDH, we used an experimental approach to inhibit EDH responses by enhancing NO-mediated responses, but a previous study (16) has shown the importance of NO for angiogenesis. Thus, further studies are needed to directly enhance or reduce EDH responses, although no approach has yet been

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established at present. Third, we did not examine the nature of EDH in the hindlimb circulation in mice, although endothelium-derived H_2O_2 has been identified as an EDH factor in mesenteric arteries of mice (20, 29). Fourth, in the present study, we did not directly show the production of excessive NO or ROS in ischemic tissue, although levels of 3-NT were significantly higher in eCav-1-KO mice compared with control mice. Fifth, in the present study, we did not evaluate the relationship between endothelial Cav-1 and the role of other NOS isoforms, such as neuronal NOS and inducible NOS, both of which are also modulated in ischemic tissue after hindlimb ischemia (30, 40). This issue remains to be examined in future studies. Last, but not least, although there is a clear sex difference in EDH-mediated responses (21), we only used male mice in the present study. Further studies are needed to address the sex difference in EDH-mediated responses and ischemic angiogenesis.

Conclusions. In summary, the present study demonstrates that endothelial Cav-1 plays important roles in EDH-mediated relaxations and angiogenesis in hindlimb ischemia through posttranslational protein modifications by nitrooxidative stress in mice in vivo.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.I., T.S., S.G., H. Saito, S.T., Y.I., S.K., and H. Shimokawa conceived and designed research; A.I. performed experiments; A.I. analyzed data; A.I., T.S., S.G., H. Saito, S.T., Y.I., S.K., K.S., and H. Shimokawa interpreted results of experiments; A.I., T.S., S.G., H. Saito, S.T., and H. Shimokawa prepared figures; A.I., T.S., and H. Shimokawa drafted manuscript; A.I., T.S., and H. Shimokawa edited and revised manuscript; A.I., T.S., S.G., H. Saito, S.T., Y.I., S.K., K.S., and H. Shimokawa approved final version of manuscript.

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