

## Hypoxia-augmented constriction of deep femoral artery mediated by inhibition of eNOS in smooth muscle

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<sup>1</sup>Department of Physiology, Seoul National University College of Medicine, Seoul, Korea; <sup>2</sup>Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul, Korea; <sup>3</sup>Department of Anesthesiology-Pain Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Suwon, Korea; and <sup>4</sup>Department of Oral Biology, Yonsei University College of Dentistry, Seoul, Korea

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**Han JA, Seo EY, Kim HJ, Park SJ, Yoo HY, Kim JY, Shin DM, Kim JK, Zhang YH, Kim SJ.** Hypoxia-augmented constriction of deep femoral artery mediated by inhibition of eNOS in smooth muscle. *Am J Physiol Cell Physiol* 304: C78–C88, 2013. First published October 24, 2012; doi:10.1152/ajpcell.00176.2012.—In contrast to the conventional belief that systemic arteries dilate under hypoxia, we found that  $\alpha$ -adrenergic contraction of rat deep femoral artery (DFA) is largely augmented by hypoxia ( $HVC_{DFA}$ ) while hypoxia (3%  $PO_2$ ) alone had no effect.  $HVC_{DFA}$  was consistently observed in both endothelium-intact and -denuded vessels with partial pretone by phenylephrine (PhE) or by other conditions (e.g.,  $K^+$  channel blocker). Patch-clamp study showed no change in the membrane conductance of DFA myocytes by hypoxia. The RhoA-kinase inhibitor Y27632 attenuated  $HVC_{DFA}$ . The nitric oxide synthase inhibitor [nitro-L-arginine methyl ester (L-NAME)] and soluble guanylate cyclase inhibitor [oxadiazole quinoxalin (ODQ)] strongly augmented the PhE-pretone, while neither of the agents had effect without pretone. NADPH oxidase type 4 (NOX4) inhibitors (diphenylene iodonium and plumbagin) also potentiated PhE-pretone, which was reversed by NO donor. No additive  $HVC_{DFA}$  was observed under the pretreatment with L-NAME, ODQ, or plumbagin. Western blot and immunohistochemistry analysis showed that both NOX4 and endothelial nitric oxide synthase (eNOS) are expressed in smooth muscle layer of DFA. Various mitochondria inhibitors (rotenone, myxothiazol, and cyanide) prevented  $HVC_{DFA}$ . From the pharmacological data, as a mechanism for  $HVC_{DFA}$ , we suggest hypoxic inhibition of eNOS in myocytes. The putative role of NOX4 and mitochondria requires further investigation. The  $HVC_{DFA}$  may prevent imbalance between cardiac output and skeletal blood flow under emergent hypoxia combined with increased sympathetic tone.

hypoxia/anoxia; arteries; smooth muscle; NADPH oxidase; nitric oxide synthase

MULTIPLE REGULATORY MECHANISMS play roles matching tissue perfusion and its metabolic needs. In skeletal muscle, the change of blood flow rate in response to the muscular activity is very high; 20- to 30-fold increases in blood flow rate/tissue weight (25). During sustained heavy exercise, whole body exercise capacity is critically dependent on the maximal rate of oxygen delivery to tissues. However, considering the limited volume of effective arterial circulation and relatively large mass of skeletal muscles, general vasodilation of skeletal arteries would be a potential threat to blood pressure regula-

tion, potentially leading to hypoperfusion of vital organs (7). Therefore, it is postulated that a partial vasoconstriction of skeletal arteries during strenuous whole body exercise (increased sympathetic neural activity with hypoxic condition) might occur, especially in the functionally less active muscular area (14, 16). However, it has not been yet rigorously investigated whether and how hypoxic conditions combined with sympathetic stimulation regulate the contractility of skeletal arteries.

It is generally accepted that hypoxia induces acute vasorelaxation (HVR) of systemic arteries such as coronary and renal arteries (33, 34, 36). A video analysis of cremaster arteries in situ, a popular model for skeletal microcirculation, demonstrated hypoxic vasodilation (19). Isometric contraction studies of chicken femoral arteries reported prominent HVR (36). However, this hypoxic effect was observed on top of the full contraction induced by 80 mM KCl, which might have preferentially allowed vasorelaxation (36).

Marked decreases of blood flow and luminal diameter were observed in gastrocnemius arteries in an in vivo human study during severe hypoxemia (3). In fact, previous isometric tension studies using dog femoral arteries demonstrated that anoxia induces constriction (9, 24). Such varied results indicate that the hypoxic regulation of skeletal artery requires further investigation especially regarding the level of hypoxia and accompanying pretone conditions.

We previously characterized the deep femoral artery (DFA; diameter: 150–200  $\mu$ m) of rats as a model of small feeding artery having prominent myogenic tone at physiological luminal pressure (40–100 mmHg; Refs. 2, 15). In a pilot study using DFA, we found that hypoxia ( $PO_2$ , 3–6%) consistently induces strong vasoconstriction when pretreated with phenylephrine (PhE), an  $\alpha$ -adrenergic agonist. Since the hypoxic augmentation of DFA contraction ( $HVC_{DFA}$ ) might have a physiological action of redirecting skeletal blood flow to active muscles under systemic hypoxia, we investigated its properties and underlying mechanisms.

### MATERIALS AND METHODS

*Preparation of DFAs and isometric tension measurement.* This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and also conforms to Seoul National University College of Medicine guidelines for the care and use of animals (Institutional Review Board Approval No. SNU-100209-1). Male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (60–100 mg/kg ip). After full anesthesia

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was confirmed, rats were quickly killed by cervical dislocation, and their proximal hindlimbs were dissected in ice-cold normal Tyrode's (NT) solution for the isolation of DFA. The DFA used in this study was a segment of skeletal artery located between the adductor magnus and gastrocnemius (15). In some rats, coronary arteries, middle cerebral arteries, and renal arteries were also dissected to be compared with DFA. The NT solution contained the following (in mmol/l): 140 NaCl, 5.4 KCl, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, 10 HEPES, 10 glucose, 1 CaCl<sub>2</sub>, and 1 MgCl<sub>2</sub>, adjusted to pH 7.4 with NaOH.

A segment of artery was mounted on two 25- $\mu$ m wires in a Mulvany-type myograph (Myo-Interface model 410A; DMT, Aarhus, Denmark) and stabilized in physiological salt solution (PSS) continuously gassed with 74% N<sub>2</sub>-21% O<sub>2</sub>-5% CO<sub>2</sub> at 37°C. The PSS used in myograph experiment consisted of the following (in mM): 118 NaCl, 4 KCl, 0.44 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub>, and 5.6 glucose. Before the experiments, the viability of the arteries was evaluated by a strong contraction in response to 80 mM KCl-PSS (80-K contraction). The 80 mM KCl-PSS were prepared by equimolar

substitution with NaCl in PSS. To induce hypoxic conditions, the O<sub>2</sub> fraction of bubbling gas was lowered to 3% O<sub>2</sub> by substitution with N<sub>2</sub>. PO<sub>2</sub> (%) was measured using a micro-oxygen electrode (MI-730; Microelectrodes, Bedford, NH) to confirm that the PO<sub>2</sub> (%) was actually lowered to 3%.

**Western blot analysis.** Dissected DFAs were washed with ice-cold PBS and lysed with a lysis buffer [50 mM Tris-HCl pH 7.4, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche, Germany)]. Lysates were homogenized in a dounce homogenizer on ice. Extracts were clarified by centrifugation at 13,000 g for 15 min at 4°C. Supernatants were denatured using 5 $\times$  SDS loading buffer and separated by gel electrophoresis on a 6.5% SDS-polyacrylamide gel. The separated proteins were transferred to a PVDF, and the protein levels were detected using following primary antibodies; rabbit anti-NOX4 (EPITOMICS, Burlingame, CA), mouse anti-endothelial nitric oxide synthase (eNOS) (BD Biosciences San Diego, CA), and rabbit anti-inducible nitric oxide synthase (iNOS) (NOVUS Biologicals, Littleton, CO). Bands were visualized using a

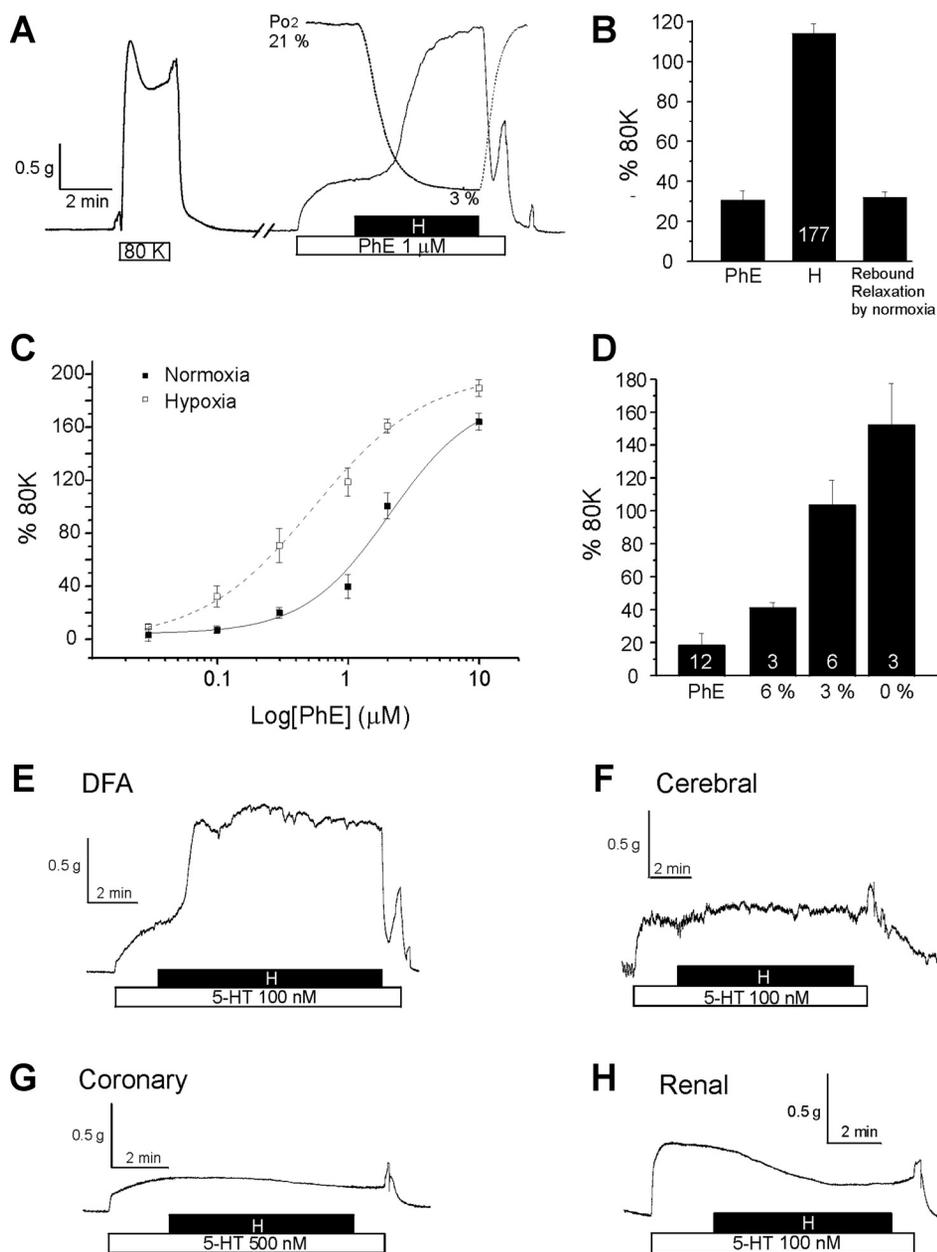


Fig. 1. Augmentation of partial contraction by acute hypoxia in deep femoral artery (DFA). *A*: representative trace of hypoxic vasoconstriction of deep femoral artery (HVC<sub>DFA</sub>). In each vessel, after confirming depolarization-induced contraction by 80 mM KCl (80 K), a partial pretone was induced by 1  $\mu$ M phenylephrine (PhE). Additional hypoxia (3% PO<sub>2</sub>, H) induced sustained contraction that was equivalent to 80-K contraction. On reoxygenation, a transient relaxation and phasic contraction were observed. *B*: summary of DFA tones under PhE pretreatment, additional hypoxia, and reoxygenation (N) that were normalized to the 80-K contraction (%80 K); 177 arteries (DFA) were tested (number in bars). Throughout Figs. 2–10, numbers of subjects are indicated in the bars. *C*: concentration-dependent contractions of DFA by PhE under normoxia and hypoxia ( $n = 5$ –7). *D*: comparison of HVC<sub>DFA</sub> induced by various PO<sub>2</sub> (6, 3, and 0%). *E*–*H*: effects of hypoxia (3% PO<sub>2</sub>) on serotonin (5-HT)-induced pretone in various systemic arteries (deep femoral, cerebral, coronary, and renal arteries). Representative traces among 5 cases for each type of artery are shown.

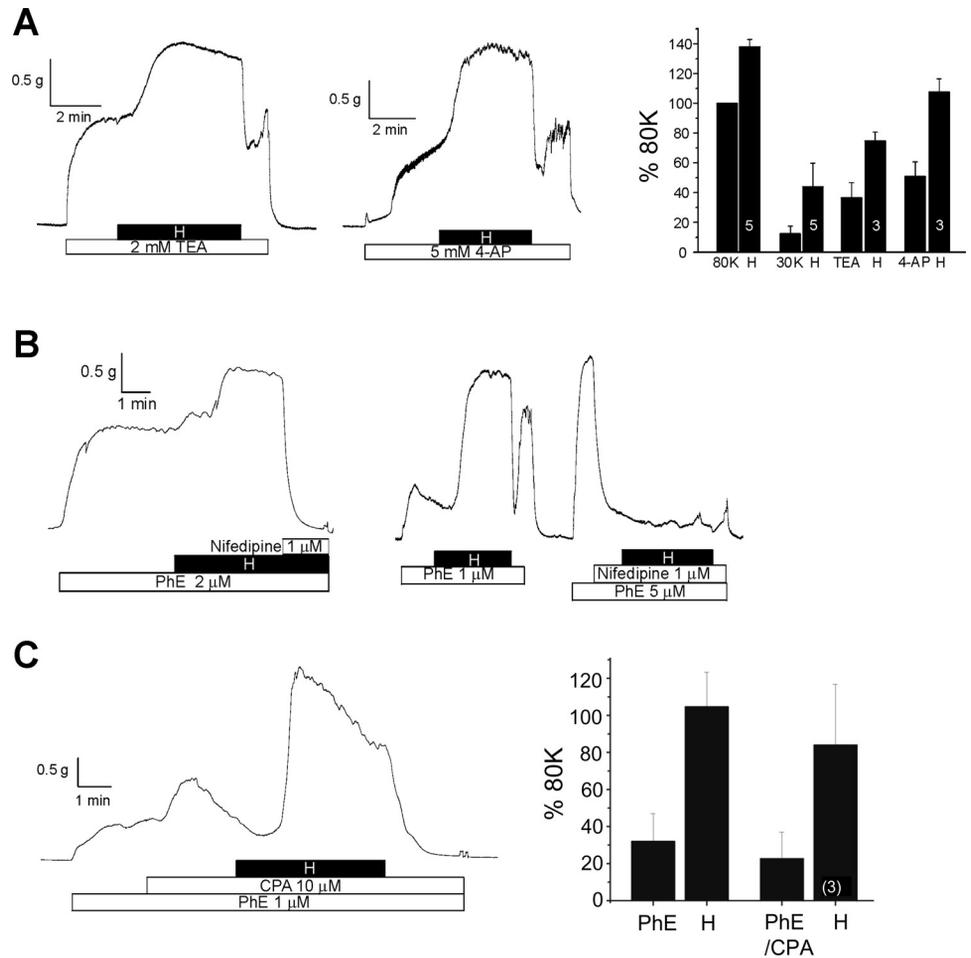


Fig. 2. Requirement of voltage-gated L-type  $Ca^{2+}$  channels for  $HVC_{DFA}$ . *A*: pretone conditions induced by  $K^+$  channel blockers [tetraethylammonium (TEA) and 4-aminopyridine (4-AP)] were also effective for  $HVC_{DFA}$  (left). In addition, high K (30 and 80 mM)-induced contractions were also augmented by hypoxia as summarized in bars at right. *B*:  $HVC_{DFA}$  was completely inhibited by nifedipine (1  $\mu$ M L-type  $Ca^{2+}$  channel blocker) applied before as well as after hypoxia. *C*:  $HVC_{DFA}$  was still observed after depleting  $Ca^{2+}$  stores in sarcoplasmic reticulum by the applying sarco(endo)plasmic reticulum  $Ca^{2+}$ -ATPase inhibitor cyclopiazonic acid (CPA; 10  $\mu$ M). Effects of CPA pretreatment on  $HVC_{DFA}$  are summarized in bars at right.

horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Millipore, Darmstadt, Germany) and HRP-conjugated anti-mouse IgG (Cell Signaling, Danvers, MA) in conjunction with SuperSignal West Pico. (Thermo Scientific, Meridian, MS). As positive control for the expression of NOX4 and eNOS, cardiac muscle was used. For iNOS, HEK 293 cells were transfected with human iNOS cDNA using FuGEN-6 (Roche Applied Science, Seoul, Korea).

**Immunohistochemistry.** Tissues from the deep femoral artery were fixed in 4% paraformaldehyde, paraffin embedded, and then cut into sections. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min at room temperature. Primary antibodies were diluted in antibody diluents (Invitrogen, Grand Island, NY): monoclonal rabbit anti-NOX4 (EPITOMICS), monoclonal mouse anti-eNOS (BD Biosciences), and polyclonal rabbit anti-iNOS (NOVUS Biologicals). Secondary antibodies were used in Dako Envision+System-HRP labeled (Dako, Glostrup, Denmark) polymer anti-rabbit (or anti-mouse). For the chromogenic reaction, we used in DAB chromogen and substrate buffer for 10 min at room temperature. Finally, the sections were washed in distilled water for 5 min, counterstained with Mayer's hematoxylin for 15 s, washed again in distilled water, dehydrated with ethanol, cleared with xylene, and mounted.

**Preparation of isolated myocytes and patch-clamp recording.** DFA myocytes were isolated and applied for patch-clamp study as described previously (15). Briefly, DFAs were incubated in  $Ca^{2+}$ -free NT with 1 mg/ml papain (10–15 min) and 3 mg/ml collagenase (10–15 min). Tissue chunks were gently agitated with Pasteur pipette, and the isolated myocytes were transferred into a bath mounted on the stage of an inverted microscope with perfused bath (0.2 ml, at

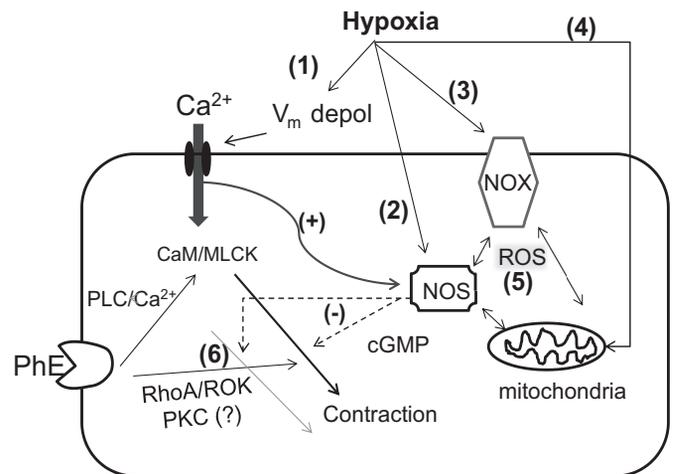


Fig. 3. Schematic model of key steps regulating the smooth muscle contractility and hypothetical  $O_2$ -sensitive paths for  $HVC_{DFA}$ . Numbers in parentheses are the putative hypoxia-sensing sites where the pharmacological agents and electrophysiological test were exerted in this study. 1) depolarizing conductance change, 2) nitric oxide synthase (NOS), 3) NADPH oxidase (NOX), 4) mitochondria electron transport chain (mETC), 5) reactive oxygen species (ROS) microenvironment affected by multiple factors, and 6);  $Ca^{2+}$ -sensitizing mechanisms via RhoA/Rho-kinase (ROK) and/or protein kinase C (PKC).

5 ml/min). Whole cell patch-clamp experiments were performed at room temperature (22–25°C) using a patch-clamp amplifier (Axo-patch-1C; Axon Instruments, Foster City, CA) and pCLAMP software v.9.2 and Digidata-1440A (Axon Instruments). The Cs<sup>+</sup>-rich pipette solution contained the following (in mmol/l): 100 L-aspartic acid, 32 CsCl, 10 EGTA, 10 HEPES, and 5 Mg-ATP, adjusted to pH 7.25 with CsOH.

**Chemicals and solutions.** All drugs and chemicals were obtained from Sigma (St. Louis, MO).

**Statistical analysis.** Data are shown as means ± SE with number of tested arteries indicated as *n*. Paired or unpaired Student's *t*-test was used for statistical analysis, with *P* value for significant difference set at *P* < 0.05.

## RESULTS

**Basic characteristics of HVC<sub>DFA</sub>.** Isometric tone of DFA was not changed by hypoxia (<3% PO<sub>2</sub>) alone (data not shown, *n* = 15). However, under a partial pretone (20–40% of 80-K contraction) induced by PhE (0.5–1 μM), hypoxia induced additional contraction (HVC<sub>DFA</sub>) that was similar to the 80-K contraction (110 ± 5.1%; Fig. 1B). While the development of HVC<sub>DFA</sub> was relatively slow (>2 min for steady-state contraction), a rapid relaxation was consistently observed on reoxygenation in the presence of PhE (Fig. 1, A and B). The concentration-dependent contractions of DFA by PhE were shifted to both leftward and upward directions by hypoxia, indicating decreased EC<sub>50</sub> and increased maximum contraction

(Fig. 1C). Relatively mild hypoxia (PO<sub>2</sub> 6%) also induced HVC<sub>DFA</sub>, and conditions close to anoxia (bubbled with 100% N<sub>2</sub>) tended to induce larger HVC<sub>DFA</sub> than that induced by 3% PO<sub>2</sub> (Fig. 1D).

HVC<sub>DFA</sub> was also observed with serotonin-induced pretone conditions (Fig. 1E). Differing from DFA, cerebral, coronary, and renal arteries did not show HVC but showed no change or relaxation under serotonin-pretone (Fig. 1, D–F). Serotonin was used as the pretone agonist because cerebral arteries do not respond to PhE.

We also tested whether other types of pretone conditions are effective for HVC. Chemical voltage-depolarization by K<sup>+</sup> channel blockers (2 mM tetraethylammonium or 5 mM 4-aminopyridine) or by raised KCl (30 or 80 mM)-induced contractions that were also augmented by hypoxia (Fig. 2A). Hereafter, DFA was used to investigate the properties and mechanisms of HVC using 3% PO<sub>2</sub> as the standard hypoxia condition with PhE-induced partial contraction (PhE-pretone). HVC<sub>DFA</sub> was completely inhibited by L-type Ca<sup>2+</sup> channel inhibitor (nifedipine, 1 μM) either before or after hypoxia (Fig. 2B). In contrast, cyclopiazonic acid (CPA, 10 μM), an inhibitor of sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA), did not block HVC<sub>DFA</sub> (Fig. 2C; *n* = 3). We also confirmed that pretreatment with thapsigargin (2 μM), another inhibitor of SERCA, did not block HVC<sub>DFA</sub> (*n* = 2, data not shown). Because the SERCA inhibitors would impair the Ca<sup>2+</sup> storing

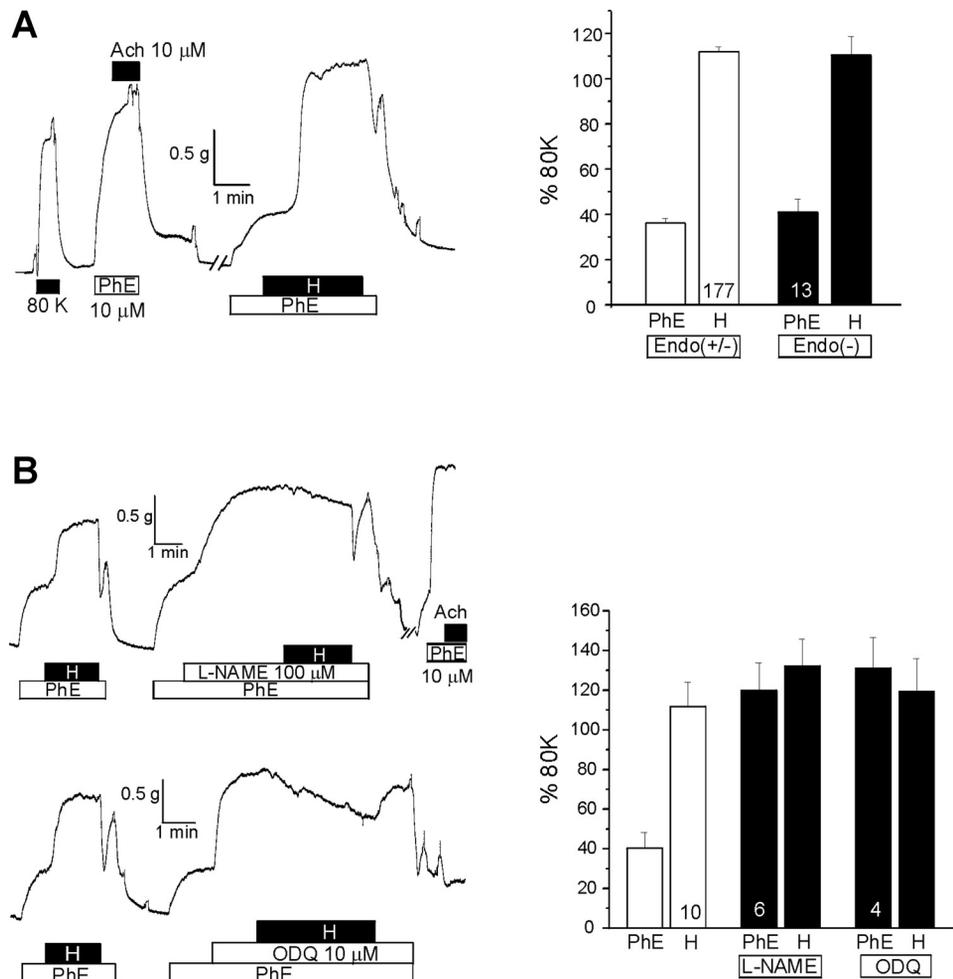


Fig. 4. Strong augmentation of PhE-pretone by NOS and soluble guanylate cyclase (sGC) inhibitors. **A:** HVC<sub>DFA</sub> was observed in the DFA where endothelium was removed by mechanical abrasion. Functional removal of endothelium was confirmed by no relaxation of DFA by ACh (left). Summary of HVC<sub>DFA</sub> in the endothelium denuded DFA (filled bars) compared with the total summary (empty bars reused from Fig. 1B). **B:** under pretreatment with PhE (1 μM), either NOS inhibitor [nitro-L-arginine methyl ester (L-NAME), 100 μM, top] or sGC inhibitor [oxadiazole quinoxalin (ODQ), 10 μM, bottom] increased the tone of DFA. Additional hypoxia had no further contraction or even partial relaxation. Summary of the PhE-pretone augmentation by NOS and sGC inhibitors (right). Control HVC<sub>DFA</sub> was also confirmed in each artery (empty bars).

function of SR, these results indicated that HVC<sub>DFA</sub> was not associated with the recruitment of SR Ca<sup>2+</sup> release. In addition, the HVC<sub>DFA</sub> was affected neither by tetrodotoxin (2 μM) nor by guanethidine (10 μM, norepinephrine release inhibitor at synaptic varicosities), excluding the indirect effect of hypoxia via perivascular sympathetic nerves (*n* = 3 and 4, respectively, data not shown).

**Mechanisms for HVC.** Because pretone was indispensable for HVC<sub>DFA</sub>, we suspected that hypoxia either augmented the intracellular signaling pathways for the contraction (Fig. 3, steps 1 and 6) or hampered the relaxing influence such as nitric oxide synthase (NOS; Fig. 3, step 2). As for the putative O<sub>2</sub> sensor of HVC<sub>DFA</sub>, both NADPH oxidase and mitochondrial enzymes with heme moiety were suggested (Fig. 3, steps 3 and 4). In the following experiments, each of the above steps was examined using pharmacological tools.

Previous studies using dog arterial strips suggested that anoxic conditions induce contraction through an endothelium-dependent release of putative vasoactive substance(s) (8, 22). Different from the previous studies, the HVC<sub>DFA</sub> in rat was consistently observed after the endothelium was removed by mechanical abrasion of lumen, which was confirmed from the absence of vasorelaxation in response to ACh (Fig. 4, A and B). Interestingly, in the absence of functional endothelium, the application of NOS inhibitor [nitro-L-arginine methyl ester (L-NAME), 100 μM] induced strong contraction of DFA with PhE-pretone, upon which no further contraction was induced by hypoxia (Fig. 4B). Application of NOS-1 (neuronal NOS) inhibitor (*S*-methyl-L-thiocitrulline, 1 μM) did not affect the PhE-pretone of DFA (data not shown; *n* = 3). No significant expression of NOS-2 (iNOS) was confirmed in DFA by immunoblot and immunohistochemistry assays (see Fig. 7). Ox-

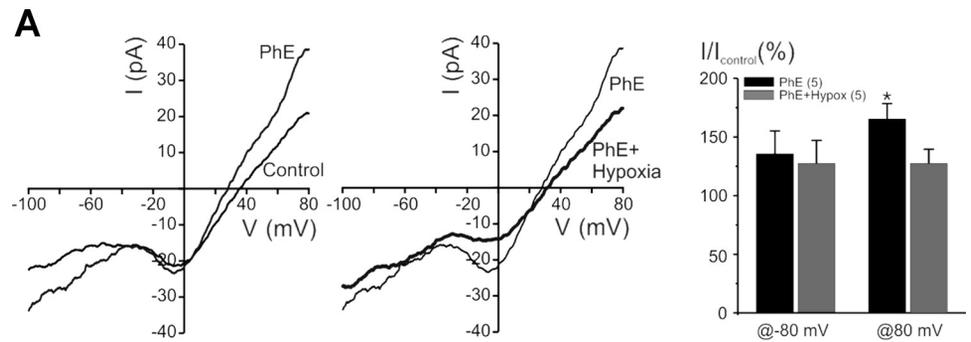
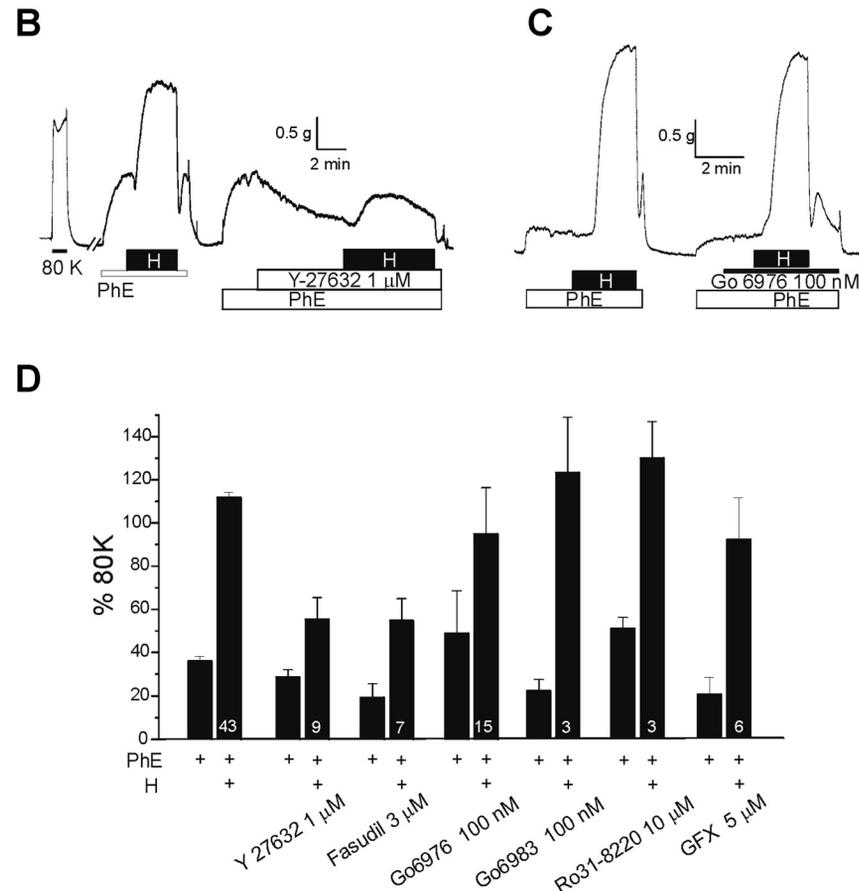


Fig. 5. Putative role of Ca<sup>2+</sup> sensitization mechanism for HVC<sub>DFA</sub>. **A:** current to voltage relation (*I/V* curves) obtained by applying ramp-like depolarization (from -100 to 80 mV) under the whole cell patch-clamp recording using Cs<sup>+</sup>-rich pipette solution. Representative *I/V* curves obtained in a DFA myocyte are shown. Summary of normalized current amplitudes (*I/I*<sub>control</sub> %) at -80 and 80 mV is shown as bars at right (*n* = 5). PhE (1 μM) increased membrane conductance (slope of *I/V* curve, left). Additional hypoxia did not show significant effect on the conductance (middle). \**P* < 0.05. **B:** representative trace of arterial tone showing inhibitory effect of RhoA/ROK inhibitor (1 μM Y-27632) on HVC<sub>DFA</sub>. **C:** no effect of PKC inhibitor (0.1 μM Go6976) on HVC<sub>DFA</sub>. **D:** summary of the effects of ROK inhibitors (Y-27632 and fasudil) and PKC inhibitors (Go6976, Go6983, Ro31-8220, and GFX) on HVC<sub>DFA</sub>.



adiazole quinoxalin (ODQ; 10  $\mu\text{M}$ ), a specific inhibitor of soluble guanylate cyclase (sGC), also markedly augmented the PhE-pretone of DFA where additional hypoxia induced slight relaxation (Fig. 4B). The lack of HVC in the presence of NOS/sGC inhibitors was not due to the limit of contractility because the maximum inducible tone of DFA was  $>180\%$  of 80-K contraction (Fig. 1C). A treatment with L-NAME or ODQ alone did not affect the tone of DFA (data not shown;  $n = 5$ , respectively). Such results suggested that NOS-3 (eNOS) and cGMP-dependent vasorelaxation was simultaneously induced under the PhE-pretone and hypoxia might have suppressed the vasorelaxing influence from eNOS/sGC activity.

Since  $\text{HVC}_{\text{DFA}}$  was completely inhibited by nifedipine (Fig. 2B), electrophysiological changes (e.g., activation of nonselective cationic current in DFA myocyte; Fig. 3, *step 1*) were suggested for  $\text{HVC}_{\text{DFA}}$ . Under the whole cell voltage clamp with CsCl pipette solution, ramp-like depolarization was applied to obtain current-voltage relation (*I/V* curve). A pretreatment with 1  $\mu\text{M}$  PhE slightly increased the membrane conductance. However, additional hypoxia had no effect on the membrane conductance of DFA myocytes (Fig. 5A).

The level of smooth muscle contraction is determined by phosphorylation states of myosin light chain ( $\text{MLC}_{20}\text{-P}$ ), which is affected not only by  $\text{Ca}^{2+}$ /calmodulin and MLC kinase but also by MLC phosphatase under the regulation by RhoA-

dependent kinase (RhoA/ROK) or PKC. Therefore, we investigated whether the “ $\text{Ca}^{2+}$  sensitization” mechanisms play a role in  $\text{HVC}_{\text{DFA}}$ . ROK inhibitor (1  $\mu\text{M}$  Y27632 or 3  $\mu\text{M}$  fasudil) significantly attenuated  $\text{HVC}_{\text{DFA}}$  (Fig. 5, B and D). In contrast, none of the PKC inhibitors (Go6976, Go6983, Ro 31-8220, and GFX) affected  $\text{HVC}_{\text{DFA}}$  (Fig. 5, C and D). Taken together, the above results suggested that a hypoxic inhibition of NOS activity and subsequent recovery of  $\text{Ca}^{2+}$  sensitivity of myocytes (Fig. 2, *steps 2* and *6*) could explain the  $\text{HVC}_{\text{DFA}}$  rather than the electrophysiological changes.

*O<sub>2</sub>-sensing pathway for HVC.* As possible physiological  $\text{O}_2$  sensors in acute hypoxic responses, hemoproteins such as NADPH oxidase (NOX) and cytochrome-associated proteins in the mitochondrial electron transport chain process (mETC) have been suggested in carotid body and pulmonary artery smooth muscles (1, 18, 20, 28, 26, 31, 34). Diphenylene iodonium (DPI, 10  $\mu\text{M}$ ), a nonspecific inhibitor of various flavoproteins including NOX and mitochondrial cytochrome oxidase, augmented the PhE-pretone of DFA (Fig. 6, A and D). Interestingly, additional hypoxia slowly reversed the DPI-induced contraction (Fig. 6A). Plumbagin has been recently presented as a NOX4-specific inhibitor (10) and was applied in a recent study for hypoxic pulmonary vasoconstriction (1). Similar to the effect of DPI, plumbagin (5  $\mu\text{M}$ ) induced strong contraction of DFA with PhE-pretone, and the additional

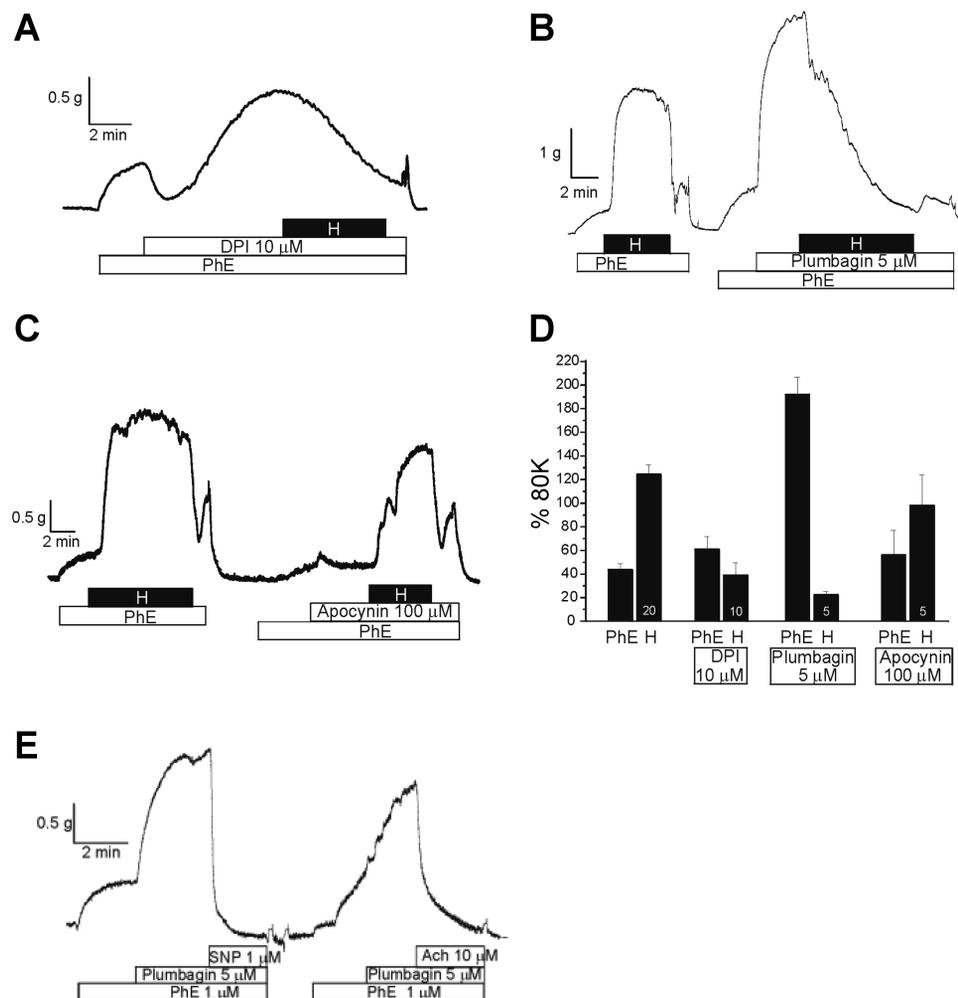


Fig. 6. Effects of NOX inhibitors on  $\text{HVC}_{\text{DFA}}$  and expression of NOX4 and NOS-3 in DFA myocytes. A and B: both DPI (10  $\mu\text{M}$ ; A) and plumbagin (5  $\mu\text{M}$ ; B) augmented PhE-pretone in DFA. Additional hypoxia induced relaxation instead of  $\text{HPV}_{\text{DFA}}$ . C: apocynin (100  $\mu\text{M}$ ) did not augment PhE-pretone, and additional hypoxia induced  $\text{HVC}_{\text{DFA}}$  with reduced amplitude. D: summary of the effects of NOX inhibitors on DFA tone and  $\text{HVC}_{\text{DFA}}$ . E: plumbagin-induced augmented PhE-pretone was completely reversed by the NO donor [sodium nitroprusside (SNP), 1  $\mu\text{M}$ ] or by the endothelial NO releasing agonist (ACh, 10  $\mu\text{M}$ ).

hypoxia induced vasorelaxation (Fig. 6, *B* and *D*). While the above results suggested that an altered activity of NOX under hypoxia might be involved,  $HVC_{DFA}$  was consistently observed in the presence of apocynin (500  $\mu$ M), which is known to inhibit the formation of active NOX2 complex between p47 and gp91phox (Fig. 6, *C* and *D*) (30).

The mimicry of  $HVC_{DFA}$  (augmentation of PhE-pretone) by plumbagin (NOX4 inhibitor) and NOS/sGC inhibitors (L-NAME and ODQ) suggested that  $O_2$ -dependent regulations of NOX4 and NOS/sGC might underlie the  $O_2$ -sensory mechanism. The plumbagin/PhE-induced strong contraction was completely reversed by the NO donor sodium nitroprusside (10  $\mu$ M)

or by ACh in the endothelium-intact DFAs (Fig. 6*E*) suggesting that neither eNOS activation nor NO-dependent cGMP production was nonspecifically inhibited by the treatment with plumbagin. The expressions of eNOS (NOS-3) and NOX4, but not iNOS (NOS-2) expression, were confirmed by immunoblot assay in the endothelium-denuded DFA (Fig. 7*A*). Also, more specific localization of eNOS and NOX4 was investigated in the immunohistochemistry assay of intact DFA (Fig. 7*B*). Both eNOS and NOX4 were strongly expressed in the endothelium. However, although relatively faint, the expression of eNOS and NOX4 in the smooth muscle layer was observed compared with the absence of iNOS signal (Fig. 7*B*).

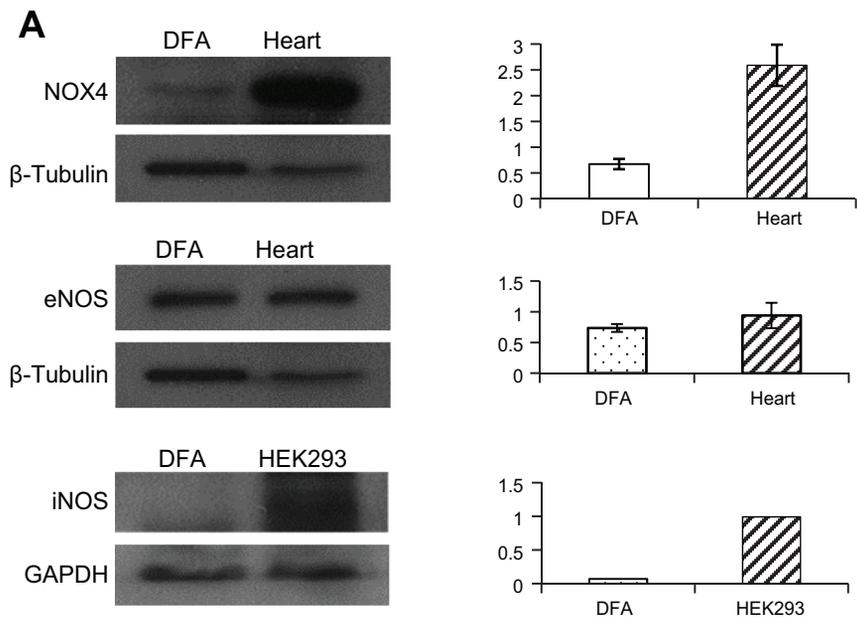


Fig. 7. Immunoblot and immunohistochemistry assays for NOX4, endothelial NOS (eNOS), and inducible NOS (iNOS) in DFA. *A*: representative cases of immunoblot assays showing the expression of NOX4 and eNOS while no significant expression of iNOS in the endothelium abraded DFA. As for the positive control, the results of cardiomyocytes (NOX4 and eNOS) and HEK293 cells (iNOS) are shown. Summary of the normalized expression against  $\beta$ -actin (NOX4 and eNOS) or GAPDH (iNOS) is shown in the bars at right. *B*: immunohistochemistry of endothelium intact DFA for NOX4, eNOS, and iNOS. The expression of NOX4 and eNOS (brown color) in endothelium is obvious while faint expressions of both proteins are also confirmed in the smooth muscle layer. In contrast, iNOS was expressed neither in the endothelium nor in the smooth muscle.

mETC function could be affected under hypoxia, which might underlie the  $HVC_{DFA}$ . In DFA, inhibitors of mETC [rotenone (100 nM), the complex I inhibitor myxothiazol (100  $\mu$ M), the complex III inhibitor cyanide (NaCN, 0.5 mM), and complex IV inhibitor] commonly suppressed  $HVC_{DFA}$  (Fig. 8). The inhibitory effect of rotenone was strongest among the tested agents. Different from the effects of eNOS and NOX inhibitors, none of the mETC inhibitors mimicked  $HVC_{DFA}$ .

Then, we investigated whether reactive oxygen species (ROS) scavengers or antioxidants affect  $HVC_{DFA}$ . A variety of agents were tested; tempol (5 mM), *N*-acetyl cysteine (1 mM), ascorbic acid (vitamin C, 1–10 mM), trolox (0.2 mM), tiron (1 mM), ebselen (10  $\mu$ M), and PEG-catalase (1,000 IU/ml). However, none of them mimicked or inhibited  $HVC_{DFA}$  (Fig. 9, *A* and *B*). In contrast with the ineffectiveness of ROS scavengers, an exogenous application of  $H_2O_2$  affected the DFA under pretreatment with PhE. The PhE-pretone was augmented by  $H_2O_2$  up to 20  $\mu$ M whereas attenuation at the higher concentration (Fig. 9, *D* and *E*).  $HVC_{DFA}$  was still observed with  $H_2O_2$  up to 20  $\mu$ M. However, no  $HVC_{DFA}$  occurred under the pretreatment with 50  $\mu$ M  $H_2O_2$  (Fig. 9, *D* and *E*).

## DISCUSSION

In this study, we first report the HVC in rat skeletal arteries ( $HVC_{DFA}$ ). The hypoxia-induced or -augmented vasoconstriction has been extensively investigated in pulmonary arteries, called hypoxic HPV. From previous studies on the cellular mechanisms of HPV, it is generally believed that the pulmonary arterial smooth muscle cells are both sensors and effectors for the HPV (28, 31, 33). Because  $HVC_{DFA}$  was consistently observed in the absence of functional endothelium, the sensor for  $HVC_{DFA}$  seemed to be also equipped within the smooth muscle cells.

Previously, we found that the HPV of isolated pulmonary arteries from rats specifically requires thromboxane  $A_2$  as a pretone agent (35). Similarly, a partial pretone was also indispensable for the  $HVC_{DFA}$ . The requirement of pretone indi-

cates that hypoxia did not directly trigger constrictive signals but sensitized DFA to contraction signals or alleviated relaxing actions intrinsic to DFA.

$O_2$ -sensing mechanisms of vascular myocytes have been most extensively investigated in pulmonary arterial smooth muscle cells, and those mechanisms might be categorized into 1)  $O_2$ -sensitive ion channel hypotheses and 2) ROS-mediated regulation of  $Ca^{2+}$  sensitization. Each category can be further divided according to specific molecule(s) claimed by research groups. As for the underlying mechanisms of  $HVC_{DFA}$ , we initially suspected that hypoxia induced depolarization of myocytes because the HVC was completely suppressed by the L-type voltage-operated calcium channel (VOCC<sub>L</sub>) inhibitor nifedipine (Fig. 2*B*). However, since the membrane conductance of DFA myocytes was not increased by hypoxia (Fig. 5*A*), our interpretation is that hypoxia did not directly augment membrane depolarization. Consistently,  $HVC_{DFA}$  was still observed under the chemical voltage clamp conditions like  $K^+$  channel blocker and high  $K^+$  bath solution (Fig. 2*A*). This suggests that hypoxia augments the contractions induced by various conditions such as VOCC<sub>L</sub> activation. In this respect, the complete inhibition of  $HVC_{DFA}$  by nifedipine could be interpreted as blockade of the primary condition.

*Alleviation of NO-dependent vasorelaxation by hypoxia?* The strong contraction induced by L-NAME upon PhE-pretone of endothelium-denuded DFA indicated that the NOS activity in smooth muscle might partly attenuate the vascular tone induced by PhE (Fig. 4). No significant HVC in the presence of L-NAME suggested that NOS might have been also targeted by hypoxia (Fig. 4*B*). Since the maximum tone of DFA could reach to 200% of 80-K contraction (Fig. 1*C*), the lack of HVC upon L-NAME-induced tone (120% of 80-K contraction) was not likely due to the limit of contractility. Although conventional wisdom was that only endothelial cells express NOS (i.e., NOS3), recent studies have shown that arterial myocytes also express multiple subtypes of NOS (5). Since a pretreatment with NOS1 (neuronal NOS)-specific inhibitor (SMTC)

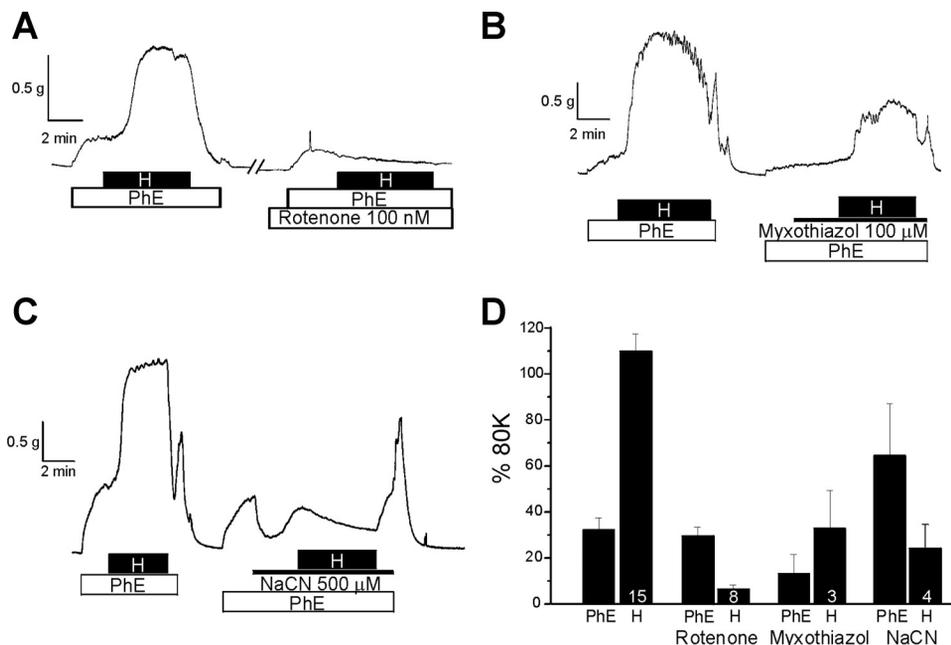


Fig. 8. Effects of mETC inhibitors on  $HVC_{DFA}$ . *A–C*: representative traces of  $HVC_{DFA}$  under treatment with rotenone (100 nM), myxothiazol (100  $\mu$ M), and NaCN (500  $\mu$ M). Rotenone and NaCN abolished  $HVC_{DFA}$ , and myxothiazol decreased the amplitude of  $HVC_{DFA}$ . *D*: summary of the effects of mETC inhibitors on  $HVC_{DFA}$ .

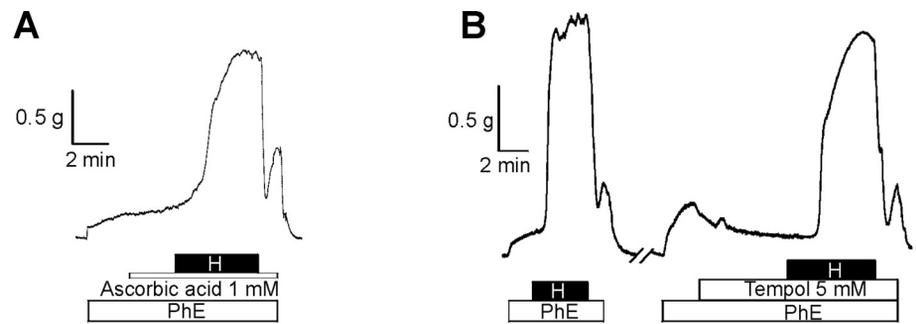
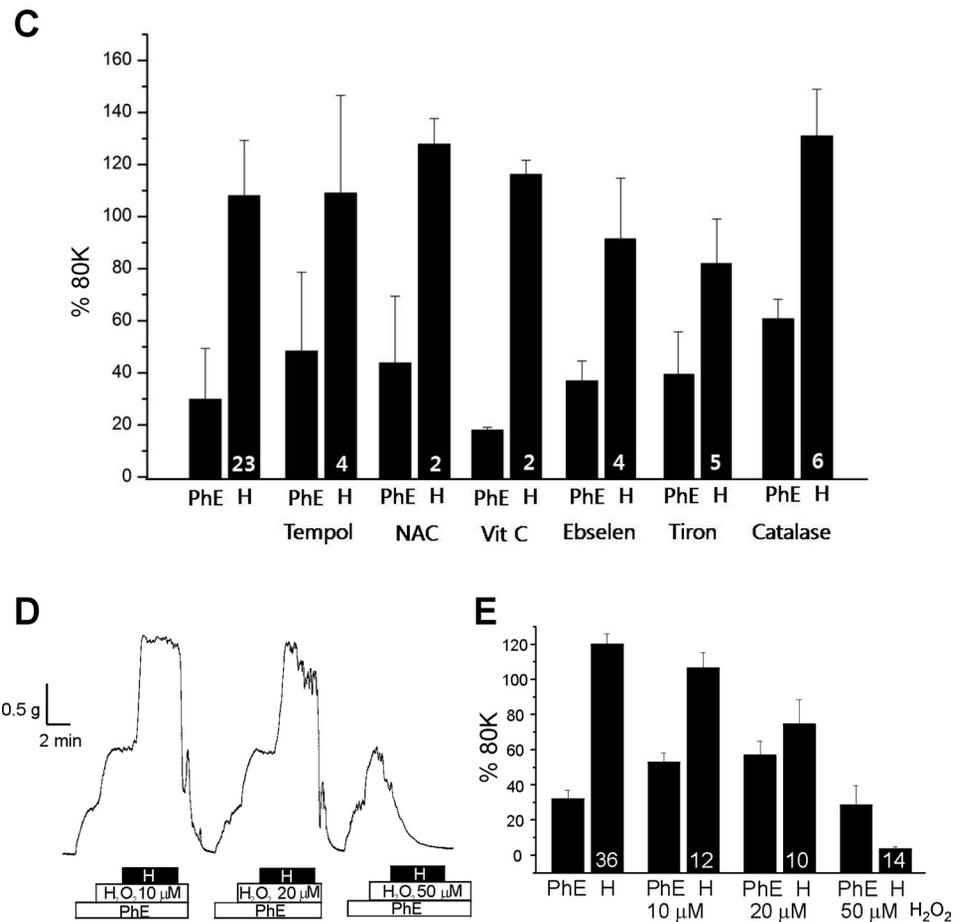


Fig. 9. No effects of ROS scavengers on HVC<sub>DFA</sub>. *A* and *B*: representative traces of HVC<sub>DFA</sub> under treatment with ascorbic acid (1 mM, *A*) and tempol (5 mM; *B*). *C*: summary of the effects of various ROS scavengers [0.2 mM tempol, 1 mM *N*-acetyl cysteine (NAC), 1 mM vitamin C (Vit C), 10 μM ebselen, 1 mM tiron, and 1,000 IU/ml PEG-catalase] on HVC<sub>DFA</sub>. Number of tested vessels is indicated in each bar. *D*: effects of H<sub>2</sub>O<sub>2</sub> on DFA tone and HVC<sub>DFA</sub>. Application of 10 and 20 μM H<sub>2</sub>O<sub>2</sub>-induced tonic contraction while 50 μM H<sub>2</sub>O<sub>2</sub> induced a transient contraction on top of PhE-pretone. Interestingly, hypoxia still induced HVC<sub>DFA</sub> with 10 and 20 μM H<sub>2</sub>O<sub>2</sub> whereas hypoxic relaxation was observed with 50 μM H<sub>2</sub>O<sub>2</sub> in the same vessel. *E*: summary of the effects of various concentrations of H<sub>2</sub>O<sub>2</sub> on PhE-pretone and HVC<sub>DFA</sub>. Number of tested vessels is indicated in each bar.



had no effect on DFA contractility and the HVC<sub>DFA</sub> (data not shown), NOS3 (eNOS) or NOS2 (iNOS) could be an important player for HVC<sub>DFA</sub>. Both immunoblot assay and immunohistochemistry assays indicated the expression of eNOS, although relatively faint, in the medial layer of DFA (Fig. 7). Because eNOS is activated by Ca<sup>2+</sup>/calmodulin-dependent mechanism, it is supposed that the pretone conditions would activate the weakly expressed eNOS in the myocytes as well as predominant Ca<sup>2+</sup>-dependent vasoconstriction mechanisms. In such conditions, hypoxic inhibition of eNOS activity could significantly augment the pretone contraction (Fig. 10). The hypothetical model was also consistent with the dependence of L-NAME- and ODQ-induced contractions on pretone conditions and the sensitivity to the VOCC<sub>L</sub> inhibitor (Fig. 4). As for the mechanism of putative hypoxic inhibition of eNOS, how-

ever, experimental clues are still lacking except the indirect results obtained from pharmacological inhibitors of NOX and mETC.

DFA showed transient relaxation on reoxygenation in the presence of PhE. The transient relaxation on reoxygenation (posthypoxic relaxation) suggested either transient activation of relaxing mechanism or revelation of underlying hypoxia-induced relaxation that was outbalanced by HVC before reoxygenation. In the presence of DPI and plumbagin combined with PhE-pretone, relatively strong relaxation was induced by hypoxia instead of HVC<sub>DFA</sub>. Such results also suggest dual effects of hypoxia on the tone of skeletal arteries.

*O<sub>2</sub>-sensing mechanism for HVC<sub>DFA</sub>*. Because the pharmacological agents (e.g., NOX inhibitors) used in this study have potentially nonspecific and indirect effects, one has to be very

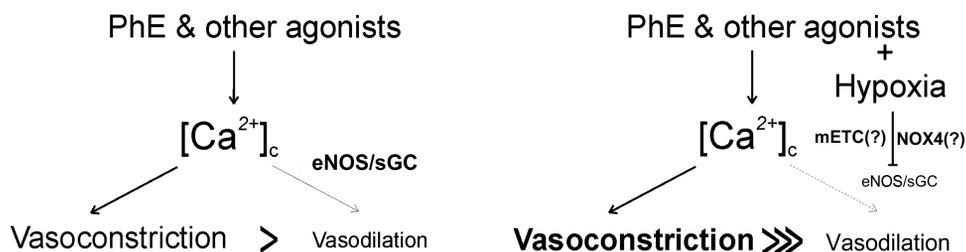


Fig. 10. Schematic model for  $HVC_{DFA}$ . In DFA, various agonists (e.g., PhE) increasing cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_c$ ) induce not only constriction but also partial relaxation owing to the stimulation of weakly expressed eNOS in the myocytes (*left*). Under hypoxia, for reasons not yet known, the eNOS/sGC signaling seems to be alleviated and the vasoconstriction is augmented (HVC; *right*). Pharmacological evidences suggest NOX4 and mETC as putative  $O_2$ -sensing mechanisms.

careful in the interpretations (28). Admitting this limitation, from the strong augmentation of PhE-pretone by DPI and plumbagin but not by apocynin, we suggested that a putative hypoxic inhibition of NOX4 might be a proximal step for  $HVC_{DFA}$ . Because a treatment with plumbagin affected neither the vasorelaxing effects of NO donor (sodium nitroprusside, 1  $\mu$ M) nor the endothelium-dependent relaxation by ACh (Fig. 6E), NOX4 or NOX4-associated molecules seem to be proximal to NOS/sGC in the signaling steps for  $HVC_{DFA}$ . In addition, this result might also suggest that plumbagin did not nonspecifically augment the contractility of DFA.

NOX4 is a constitutively active member of NOX family, and therefore, a direct effect of hypoxia on the activity of NOX4 was supposed to be a decreased production of superoxide anions ( $O_2^-$ ) that are immediately converted into  $H_2O_2$  by superoxide dismutase. In addition, it has been reported that  $H_2O_2$  stimulate eNOS (6, 29). In the presence of a relatively high concentration of  $H_2O_2$  (>20  $\mu$ M), both PhE-pretone and  $HVC_{DFA}$  was abolished (Fig. 9, D and E). Taken together, an attractive interpretation was that the hypoxic inhibition of NOX4 might weaken the  $H_2O_2$ -mediated facilitation of eNOS activity and thereby reveal the full contraction by pretone conditions, i.e.,  $HVC_{DFA}$ . Unfortunately, however, ROS scavengers did not mimic the effect of NOX4 inhibitors: the pretreatment with tempol, ebselen, tiron, and catalase only partly attenuated or had no effect on  $HVC_{DFA}$  (Fig. 9, A–C). The concentration-dependent dual effects of  $H_2O_2$  further complicated interpretations based on the model of NOX4/ $H_2O_2$ /eNOS signaling (Fig. 9, D and E). One might propose a bold hypothesis that NOX4 conveys the  $Po_2$  state to effector (e.g., eNOS) via a “nonenzymatic mechanism” without the mediating role of  $H_2O_2$ . Previously, we showed that NOX4-dependent hypoxic inhibition of two-pore domain  $K^+$  channel (TASK1) is a ROS-independent mechanism (21). However, rigorous molecular evidence is definitely required for such hypothesis, which has not accomplished yet.

Although precise mechanism is not known yet, intact mitochondrial function was indispensable for maintaining the tone of DFA as well as for  $HVC_{DFA}$ . The potent suppression of PhE-pretone and  $HVC_{DFA}$  by mETC inhibitors suggested that the mitochondria might also play as an  $O_2$  sensor for  $HVC_{DFA}$  as suggested in pulmonary and coronary arteries (12, 22). Although still controversial, the production of superoxide anion during mETC process is claimed to be increased by hypoxia (18, 20, 22). Different from  $H_2O_2$ , the increased superoxide anions could impair the NOS function as well as scavenging NO (26). The insignificant effect of ROS scaven-

gers on  $HVC_{DFA}$  might be interpretable based on the colocalization of eNOS in the outer membrane of mitochondria (13). In this respect, another hypothetical model is that spillover of superoxide anion from mETC under hypoxia might impair the eNOS/sGC activity in DFA (Fig. 10). However, fundamental limitation in the interpretation of the results with mETC inhibitors is that the impaired energy metabolism might nonspecifically affect the contractility of arteries.

*Physiological roles of HVC in skeletal arteries.* An in vivo human study demonstrated that systemic hypoxia augmented forearm vasodilation during exercise (4, 8). At a first glance, therefore,  $HVC_{DFA}$  in rat skeletal artery was counterintuitive to the physiological role of arteries perfusing muscular tissues with widely variable metabolic rates. The  $HVC_{DFA}$  phenomenon might contribute to balancing excessive vasodilation of skeletal arteries under metabolic inhibition of the adjacent tissue, especially under the tonic influence from the sympathetic nervous system. It has been recently demonstrated that constitutive activation of  $\alpha$ -adrenoreceptors in skeletal muscle can restrict the spread of dilatation within microvascular resistance networks (17).

Strenuous exercise might induce a competition between active skeletal muscles, myocardium, and central nervous system for a limited amount of maximum perfusion. A plausible role of HVC might be the maintenance of vital organ perfusion against the circulatory emergency conditions (11, 23). For example, in systemic hypoxia combined with loss of blood volume, the HVC of skeletal arteries under increased sympathetic tone would divert the limited blood flow to vital organs such as brain, heart, and kidney where no HVC was observed in their feeding arteries (Fig. 1, F–H).

In summary, we report  $HVC_{DFA}$  in rats may be mediated by the attenuation of  $Po_2$ -dependent signaling pathways of eNOS/sGC in smooth muscle. Since ROS scavengers did not completely inhibit HVC, the signaling mechanism between the candidate  $O_2$  sensors (NOX4 and mETC) and eNOS/sGC remains elusive. The HVC of skeletal arteries may prevent the imbalance between the cardiac output and the skeletal blood flow under severe exercise or under emergent hypoxia combined with increased sympathetic tone.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

## AUTHOR CONTRIBUTIONS

Author contributions: J.-A.H., E.-Y.S., H.J.K., and J.Y.K. performed experiments; J.-A.H. and D.M.S. analyzed data; J.-A.H., E.-Y.S., S.J.P., H.Y.Y., J.K.K., and Y.-H.Z. interpreted results of experiments; J.-A.H., H.J.K., and J.Y.K. prepared figures; J.-A.H., E.-Y.S., H.J.K., S.J.P., J.K.K., and Y.-H.Z. drafted manuscript; S.J.P., H.Y.Y., J.K.K., and S.J.K. conception and design of research; S.J.K. edited and revised manuscript; S.J.K. approved final version of manuscript.

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