

Vascular Hypoxic Preconditioning Relies on TRPV4-Dependent Calcium Influx and Proper Intercellular Gap Junctions Communication

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Objective—We investigated the impact of hypoxia-reoxygenation on endothelial relaxation and aimed to clarify the role of transient receptor potential cation channels V4 (TRPV4) and gap junctions in the protective effect associated with hypoxic preconditioning on the vascular function.

Methods and Results—By mimicking ischemia-reperfusion in C57BL/6 male mice *in vivo*, we documented a reduced NO-mediated relaxation and an increased endothelium-derived hyperpolarization (EDH[F])-mediated relaxation. Hypoxic preconditioning, however, restored NO relaxation and further improved the EDH(F) response. We also examined specifically 2 major effectors of the EDH(F) pathway, transient receptor potential cation channels V4 and connexins. We found that in endothelial cells, expression and activity of transient receptor potential cation channels V4 were increased by hypoxic stimuli independently of preconditioning which was interestingly associated with an increase of structural caveolar component caveolin-1 at membrane locations. Gap junctions, however, seemed to directly support EDH(F)-driven preconditioning as connexin 40 and connexin 43 expression increased and as *in vivo* carbenoxolone treatment completely inhibited the EDH(F) pathway and significantly reduced the protection afforded by preconditioning for the concomitant NO-mediated relaxation.

Conclusion—Our work provides evidence on how transient receptor potential cation channels V4 and connexins might participate in preserving vasorelaxation under hypoxia and restoring the NO-mediated pathway in hypoxic preconditioning conditions pointing out caveolae as a common signaling location. (*Arterioscler Thromb Vasc Biol.* 2012;32:2241-2249.)

Key Words: hypoxic preconditioning ■ gap junctions ■ TRPV4 ■ NO
■ endothelium-derived hyperpolarization

Treatment of ischemic heart diseases relies on an early return of blood flow to ischemic zones of the myocardium, namely the reperfusion therapy. However, reperfusion on its own has the potential to cause further irreversible myocardial cell injury and endothelial dysfunction as the consequence of a burst of reactive free radicals, such as reactive oxygen species and proinflammatory cytokines.¹ In 1986, the pioneer work of Murry et al² demonstrated that exposing the heart to transient sublethal ischemia and reperfusion protects the myocardium against functional damage and cell death caused by a subsequent sustained ischemia. This phenomenon, called ischemic preconditioning, has since proven to be true in both animals and humans³ and in many organs, including the vascular endothelium.^{4,5} With NO being the most important and best characterized endothelial factor, many studies hypothesized its involvement in the molecular cascade that leads to preconditioning in both myocardial and vascular

tissues. The group of Bolli⁶ actually proposed a mechanism where NO and oxyradical generation trigger the ischemic-preconditioning protection of porcine and rabbit heart from an episode of stunning.⁷ In the vasculature, a protective effect of ischemic preconditioning on the endothelium relaxation has also been documented to be NO-mediated.^{4,5,8} Furthermore, several authors have reported that the administration of potent NO donors, like organic nitrates,^{9,10} or drugs that enhance NO release, such as statins,¹¹ can mimic preconditioning.

In resistance arteries, however, the control of vascular tone not only depends on NO bioavailability but also on the generation of endothelium-derived hyperpolarization (EDH[F]) (previously associated with the notion of endothelium-derived hyperpolarizing factors [EDHF]). The poor characterization of the EDH(F) cascade has for a long time constituted a major drawback in the understanding of its roles. Indeed, to date very few studies have addressed the role of EDH(F)-driven

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vasodilation after ischemia-reperfusion, and the protective role of EDH(F) in ischemic preconditioning remains elusive.^{12–14} Some key mediators of EDH(F) signaling are nowadays clearly identified. Accordingly, EDH(F) is triggered by an elevation of cytoplasmic Ca^{2+} concentration in endothelial cells ($[Ca^{2+}]_i$)¹⁵ and the final opening of Ca^{2+} -activated potassium channels (K_{Ca}) expressed either on endothelium (most likely small and intermediate conductance IK_{Ca} and SK_{Ca} channels) or on smooth muscle cells (most likely large conductance BK_{Ca} channels).¹⁶ In this context, we have demonstrated the obligatory role of the transient receptor potential cation channels V4 (TRPV4) in the endothelium-dependent vascular relaxation, as the genetic deletion of these channels down regulated both the NO and EDH(F) components of the relaxation to muscarinic cholinergic stimulation.¹⁷ Also, in many resistance vessels, vascular gap junction integrity is a requisite for vascular smooth muscle cell hyperpolarization to occur.^{18,19} We have demonstrated that the EDH(F)-mediated relaxation was totally absent¹⁷ in caveolin-1 (cav-1)-deficient mice, where the expression of connexins (connexin [Cx] 43, Cx40, and Cx37) was reduced and myoendothelial gap junctions were altered. The consequences of ischemia-reperfusion and ischemic preconditioning on myoendothelial gap junctions' function in resistance arteries remain virtually unaddressed.

Thus, the aim of the present work was to investigate the impact of hypoxia and reoxygenation on endothelial relaxation and to clarify the role of 2 major effectors, namely TRPV4 channels and gap junctions, in the protective effects associated with hypoxic preconditioning on the vascular function.

Materials and Methods

Mice Models

Twelve- to 16-week-old C57BL/6 male mice were used. For hypoxia, mice were placed in a modular incubator chamber and exposed for 16 hours, to 13% O_2 and 5% CO_2 .²⁰ For preconditioning, prolonged hypoxia was preceded by 3 periods of 15-minute hypoxia (6% O_2 and 5% CO_2) followed by 15-minute reoxygenation. At the end of the procedure, mice were euthanized by cervical dislocation. Some experiments were performed on equivalent TRPV4 wild-type or knockout (KO) mice. Experimental protocols did not affect mortality rate and were approved by the local Ethics Committee according to National Care Regulations.

In Vivo Drug Administration

Carbenoxolone was administered intraperitoneally before hypoxia or preconditioning protocols; the injection volume was always 100 μ L per 10 g of body weight. Control animals received equivalent volumes of vehicle.

Measurement of Contractile Tension

Contraction and relaxation of the second branch of superior mesenteric arteries of the mouse were measured on a wire myograph as already described.¹⁷ Notably, experiments were performed in the constant presence of oxygen.

HUVEC Culture

Human umbilical vascular endothelial cells (HUVECs) were grown according to standard procedures and used between passages 3 and 5. Culture plates were incubated for various times at 37°C under normoxia (in humidified air; 5% CO_2) or under hypoxia (1% O_2 , 5% CO_2 , and 94% N_2) followed by reoxygenation. For hypoxic preconditioning, HUVECs were exposed to 2 periods of 30 minutes hypoxia

followed by 30 minutes reoxygenation and subsequent prolonged hypoxia (4 hours).

Western Blotting

Proteins from HUVECs or mouse aortas were harvested and processed for immunoblotting as described previously.²¹ Nondenaturing protein extraction was performed for endothelial nitric oxide synthase (eNOS) monomer/dimer assay and membrane extraction was used where indicated (see online-only Data Supplement). Membranes were (re) probed with antibodies against Cx43, Cx40, TRPV4 channel, phosphoserine 1177-eNOS, eNOS, and cav-1. β -actin was used as a loading control where appropriate. Peroxidase-conjugated affinity-purified goat, anti-mouse IgG, or goat anti-rabbit IgG were used as secondary antibodies.

Quantification of Cell Communication

To determine gap junction cell coupling, a flow cytometry-based method, previously described²² and validated,^{23,24} was used. Briefly, confluent HUVECs were labeled with 1 μ mol/L calcein-AM. Excess (extracellular) calcein dye was removed by triplicate washing and cells were kept in serum-free endothelial growth medium. A second cell population (dye acceptor cells) was labeled with 5 μ mol/L membrane-bound PKH26. The 2 dyes labeled cell populations were exposed to normoxia (control) or hypoxia preceded or not by preconditioning. Then, PKH26-labeled HUVECs were trypsinized and added to the calcein-labeled donor cells. Coincubation was initiated by centrifugation (15 g for 3 minutes) and continued for 90 minutes to allow for dye transfer. Loosely attached acceptor cells were removed by washing, fixed in 0.725% formalin solution, and analyzed by flow cytometry. Double staining indicated calcein dye transfer into PKH26-labeled cells.

Measurement of $[Ca^{2+}]_i$ in HUVECs

$[Ca^{2+}]_i$ was determined with the fluorescent Ca^{2+} indicator Fura-2 as previously described.¹⁷ Results were expressed as increase in fluorescence ratio (340 nm/380 nm). For additional details see the online-only Data Supplement.

Immunofluorescence Microscopy

HUVECs were grown, as previously described, to 90% confluence on gelatin-coated glass coverslips. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized with Triton X100 (0.1%), and blocked with 5% of BSA. HUVECs were then sequentially labeled with a goat anti-Cav-1 and rabbit anti-TRPV4 antibodies. Species-specific secondary antibodies (tetramethylrhodamine isothiocyanate [TRITC]-donkey anti-goat IgG and fluorescein isothiocyanate [FITC]-mouse anti-rabbit IgG) were added according to the manufacturer's protocol. Images were acquired with a Zeiss Imager.Z1 fluorescence microscope, equipped with an ApoTome device, $\times 63$ oil immersion objective lenses, and analyzed with AxioVision software (Zeiss Imager.Z1 Fluorescence Microscope, Göttingen, Germany).

Statistical Analysis

All results are expressed as mean \pm SEM. Statistical analyses were performed by Student *t* test or 1-way ANOVA followed by Bonferroni/Dunn multiple comparison test where appropriate; $P < 0.05$ was considered significant. Unmarked or ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. For Figure 2: ° $P < 0.05$; °°° $P < 0.001$ versus hypoxia.

Results

NO- and EDH(F)-Mediated Relaxation Are Differently Affected by Hypoxia-Reoxygenation and Hypoxic Preconditioning

Effect of hypoxia-reoxygenation on NO-mediated relaxation of second branch superior mesenteric arteries was determined in vessels isolated from mice exposed to hypoxia-reoxygenation in vivo. Isolated vessels were first contracted with a high-KCl

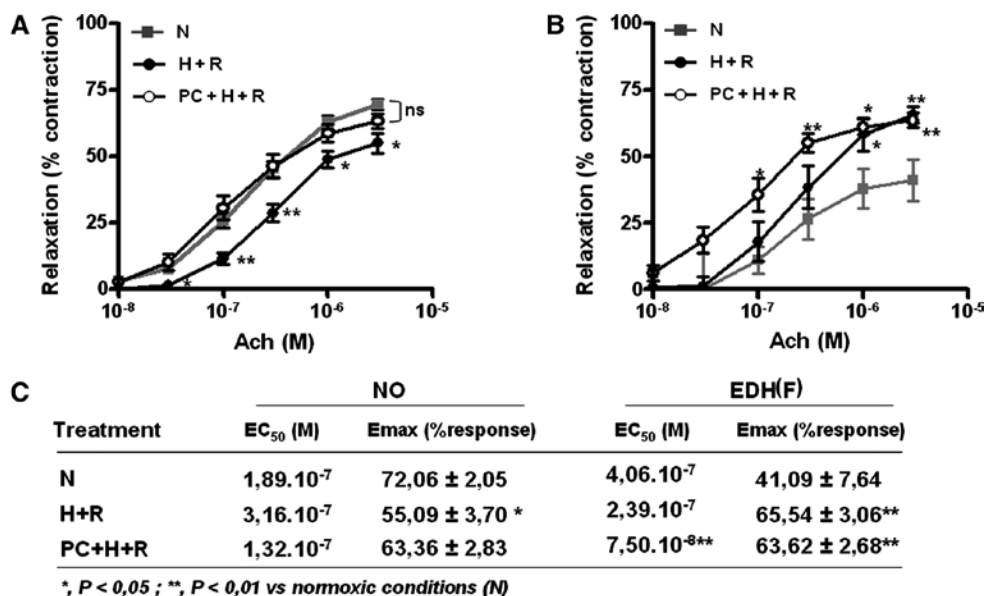


Figure 1. NO- and endothelium-derived hyperpolarization EDH(F)-mediated relaxation in mesenteric arteries isolated from mice exposed to in vivo hypoxia-reoxygenation or hypoxic preconditioning. Acetylcholine (Ach) evoked relaxation in second branch superior mesenteric arteries isolated from mice exposed to prolonged hypoxia-reoxygenation (H+R) or preconditioning followed by prolonged hypoxia-reoxygenation (PC+H+R) or normoxia (N). **A**, NO-mediated relaxation was evaluated in the presence of indomethacin (10 μmol/L) after contraction with high-KCl solution (100 mmol/L) (n=20). **B**, EDH(F)-mediated relaxation was evaluated in the presence of NG-nitro-L-arginine methyl ester (100 μmol/L) and indomethacin (10 μmol/L) after contraction with phenylephrin (1 μmol/L) (n=11). **C**, EC50 and Emax values for NO- and endothelium-derived hyperpolarization factors (EDH(F))-mediated relaxation.

solution (to exclude any EDH[F]) and exposed to acetylcholine (0.01–3 μmol/L) in the presence of 10 μmol/L indomethacin. Notably, no significant difference was observed in the contraction level of the different groups. As shown in Figure 1A, NO-mediated relaxation was significantly decreased in these arteries as compared with vessels obtained from mice kept in normoxia. To assess the effect of hypoxic preconditioning, mice were submitted to brief periods of intermittent hypoxia before prolonged hypoxia. In these conditions, the acetylcholine-dependent NO-mediated relaxation was restored.

Similarly, the effects of hypoxia-reoxygenation and preconditioning on EDH(F)-mediated relaxations were evaluated. Phenylephrine-precontracted arteries (6 μmol/L) were exposed to acetylcholine (0.01–1 μmol/L) in presence of a nitric oxide synthase inhibitor (N[G]-nitro-L-arginine methyl ester, 100 μmol/L) and indomethacin (10 μmol/L). Amplitude of the EDH(F)-evoked relaxation was significantly increased in mesenteric arteries from mice exposed to sustained hypoxia compared with control and further improved in preconditioned vessels (Figure 1B). Preconditioning also resulted in an increase in the sensitivity of the EDH(F)-mediated response to acetylcholine (EC50: 75 nmol/L, [n=8–16] versus EC50: 406 nmol/L, [n=6] for vessels isolated from normoxic mice [P=0.0032]).

Increased Gap Junction Coupling Seems Mandatory for the Protective Effect of Preconditioning on NO-Mediated Relaxation

Gap junction communications are essential for normal vascular function.²⁵ Therefore, we measured the impact of hypoxia-reoxygenation and preconditioning on the expression of their molecular components Cx40 and Cx43 in endothelial cells. We observed that although hypoxia-reoxygenation did not significantly alter the expression of both connexins, Cx40 and Cx43,

in HUVECs and mouse aortas, hypoxic preconditioning significantly increased their expression level (P=0.0060 for Cx43 and P=0.0002 for Cx40) (Figure 2A and 2B).

The functional relevance of connexins’ expression modifications was assessed by measuring the cell coupling after exposition to normoxia or hypoxia-reoxygenation preceded or not by hypoxic preconditioning. We evidenced that exposure to hypoxic preconditioning induced a significant improvement in cell communication as attested by the flow cytometry results showing a 100% increase in double-stained endothelial cells compared with control (P=0.0171) (Figure 2C).

To further investigate a potential role of gap junctions in the protection provided by hypoxic preconditioning, mice were injected intraperitoneally with the gap junction uncoupler carbenoxolone (50 mg/kg) before exposure to hypoxia preconditioning protocols. Vessels were then isolated and the NO- and EDH(F)-dependent relaxations were assessed. In vessels isolated from these mice, the EDH(F)-signaling cascade was completely abolished as attested by the total absence of EDH(F)-dependent relaxation up to 24 hours after injection of the inhibitor (not shown). Importantly, in vessels from normoxic mice, the NO-mediated relaxation to muscarinic stimulation was not significantly altered as the maximal relaxation amounted to 72±2% of contraction in control mice and to 77±6% in carbenoxolone-treated mice (Figures 1C and 2E). More interestingly, as shown in Figure 2D, carbenoxolone totally abrogated the hypoxic preconditioning-induced restoration of NO-mediated relaxation.

TRPV4 Channel Expression and Activity Are Increased by Hypoxic Preconditioning and Hypoxia-Reoxygenation

As we previously observed that Ca²⁺ influx through TRPV4 channels participates in EDH(F)- and NO-mediated relaxations,¹⁷

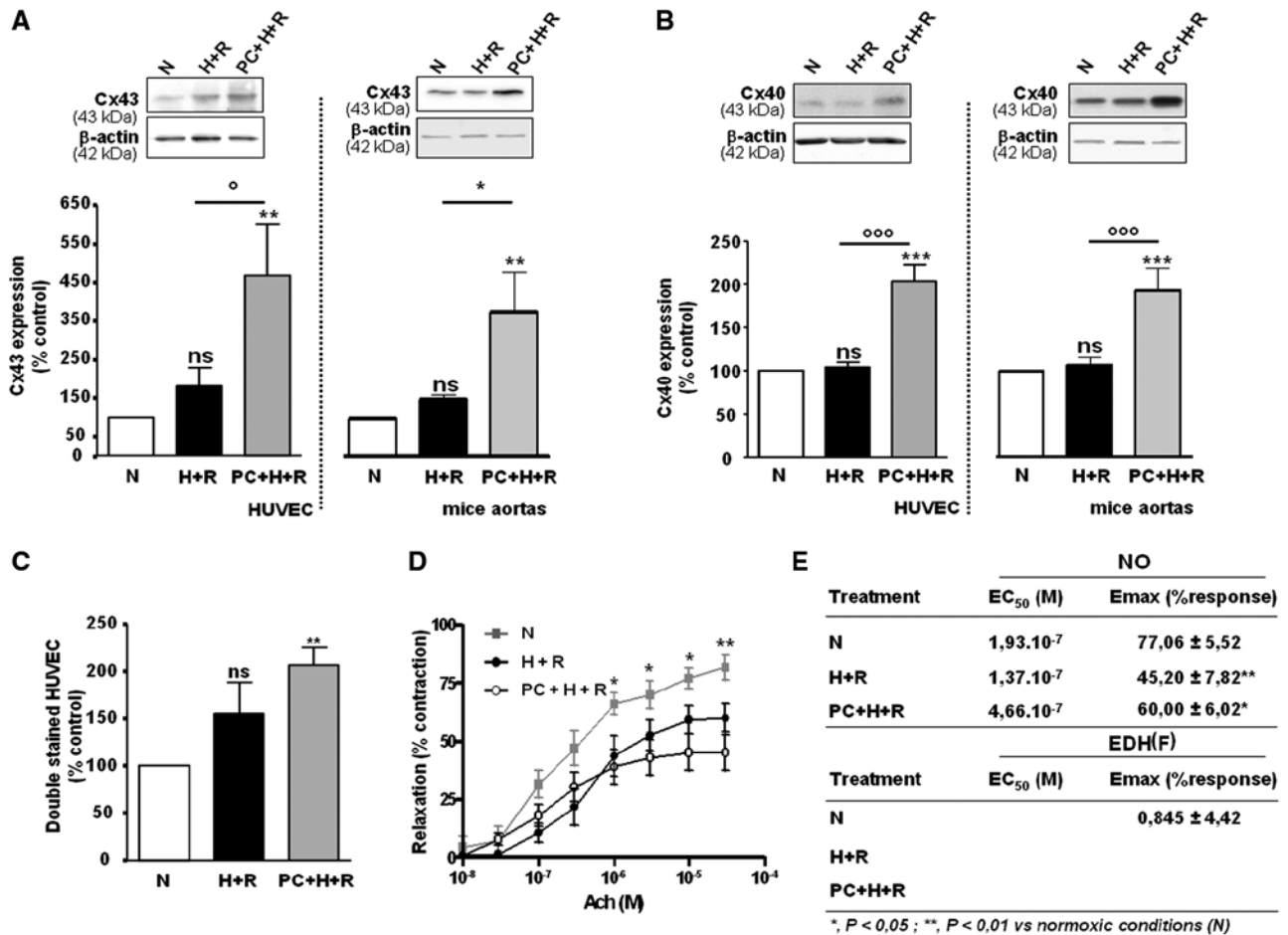


Figure 2. Involvement of gap junctions in hypoxia-reoxygenation or hypoxic preconditioning. **A** and **B**, Immunoblotting analysis for connexin (Cx) 43 and Cx40 expression in human umbilical vascular endothelial cells (HUVECs) and mouse aortas previously exposed to hypoxia-reoxygenation (H+R), hypoxic preconditioning preceding hypoxia-reoxygenation (PC+H+R) or kept in normoxia (N). β -actin was used as loading control. **C**, Gap junction communication evaluated by flow cytometry in HUVECs previously exposed to the different protocols (H+R, PC+H+R, or N). Results are expressed as a proportion of double-stained cells with PKH26 (prestaining of acceptor cells) and calcein (gap junction permeant probe). **D**, NO-mediated relaxation in second branch superior mesenteric arteries isolated from carbenoxolone-treated mice (50 mg/kg) exposed to N, H+R, or PC+H+R. Relaxing responses were induced by increasing concentrations of acetylcholine (Ach) in the presence of indomethacin (10 μ mol/L) after contraction with high-KCl solution (100 mmol/L). **E**, EC₅₀ and Emax values for NO- and endothelium-derived hyperpolarization-mediated relaxation in carbenoxolone-treated mice. ns indicates not significant; EDHF, endothelium-derived hyperpolarization factors.

we evaluated the expression and activity of these channels in endothelial cells exposed to hypoxia-reoxygenation and preconditioning. As shown in Figure 3A, both hypoxia-reoxygenation and hypoxic preconditioning increased TRPV4 channels expression in HUVECs. Notably, similar results were obtained when using protein extracts from aorta of mice exposed to similar conditions. To evaluate the possible functional consequences of these alterations on Ca²⁺ turnover, the effect of 4 α -phorbol 12,13-didecanoate (10 μ mol/L), a highly specific activator of TRPV4 channels on [Ca²⁺]_i level, was measured in HUVECs previously exposed to the different incubation protocols. Figure 3B illustrates that in cells exposed to prolonged hypoxia-reoxygenation, the Fura-2 Ca²⁺-signal increase evoked by 4 α -phorbol 12,13-didecanoate was significantly larger than that in cells kept in normoxia. Similar results were observed when cells were previously exposed to hypoxic preconditioning (Figure 3B). In these conditions, the cytosolic Ca²⁺ concentrations amounted to 50 \pm 8 nmol/L in normoxia, 116 \pm 15 nmol/L in hypoxia, and 130 \pm 22 nmol/L in hypoxic preconditioning.

We then investigated the potential involvement of TRPV4 in endothelial relaxation in hypoxic or hypoxic preconditioning conditions. We used mesenteric arteries from TRPV4 KO mice exposed to hypoxia-reoxygenation in vivo protocols in order to evaluate NO- (Figure 3C) and EDH(F)-mediated (Figure 3D) dilations. Consistent with previous data, NO- and EDH(F)-dependent relaxation were markedly depressed in normoxic TRPV4 KO mice arteries.¹⁷ However, neither hypoxia-reoxygenation nor hypoxic-preconditioning had any additional effect on NO-dependent dilation in these mice (Figure 3C). Surprisingly, exposing TRPV4 KO mice to hypoxic preconditioning before the sustained hypoxia still significantly increased the EDH(F)-evoked relaxation in mesenteric arteries, whereas hypoxia-reoxygenation did not (Figure 3D).

Effect of Hypoxia-Reoxygenation and Hypoxic Preconditioning on Cav-1 Expression, eNOS Uncoupling, and eNOS Phosphorylation

Because eNOS is the most abundantly expressed isoform of nitric oxide synthase in the endothelium and, therefore, is

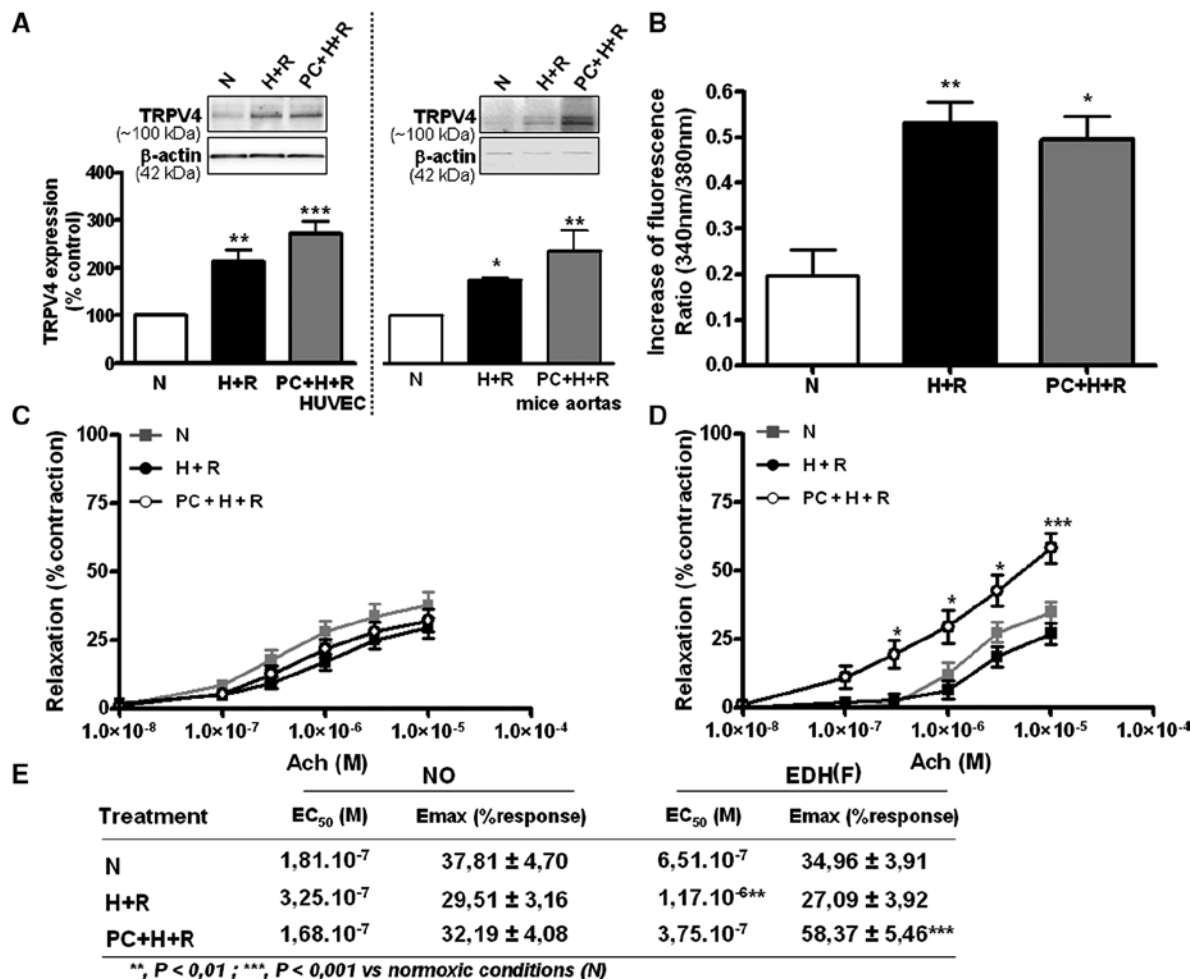


Figure 3. Transient receptor potential cation channels V4 (TRPV4) expression and channel function modulation under hypoxia-reoxygenation and hypoxic preconditioning, consequence of TRPV4 absence on endothelium-dependant relaxation. **A**, Immunoblotting analysis for TRPV4 expression in human umbilical vascular endothelial cells (HUVECs) and mouse aortas previously exposed to hypoxia-reoxygenation (H+R), hypoxic preconditioning preceding hypoxia-reoxygenation (PC+H+R), or kept in normoxia (N) (n=9). β-actin was used as loading control. **B**, Increase in [Ca²⁺]_i evoked by 4α-phorbol 12,13-didecanoate (10 μmol/L) in HUVECs submitted to N, H+R, or PC+H+R (n=3). **C** and **D**, Acetylcholine (Ach) evoked relaxation in second branch superior mesenteric arteries isolated from TRPV4 knockout mice exposed to N, H+R, or PC+H+R. NO-mediated relaxation was evaluated in the presence of indomethacin (10 μmol/L) after contraction with high-KCl solution (100 mmol/L) (**C**). Endothelium-derived hyperpolarization (EDH[F])–mediated relaxation was evaluated in the presence of NG-nitro-L-arginine methyl ester (100 μmol/L) and indomethacin (10 μmol/L) after contraction with phenylephrin (1 μmol/L)(D)(n=20). **E**, EC50 and Emax values for NO- and EDH(F)-mediated relaxation.

likely to be involved in the modified NO response observed under hypoxia and hypoxic preconditioning, we investigated some of its known regulatory mechanisms. Potential inhibitory regulation of eNOS by cav-1²⁶ was assessed by Western blotting in HUVECs after the different hypoxic protocols. Expression of cav-1 measured on total cellular homogenates did not show any influence of hypoxia-reoxygenation or preconditioning (not shown). However, when measuring the abundance of cav-1 in membrane extracts, we observed a significant increase in cav-1 expression in endothelial cells submitted to hypoxia-reoxygenation or preconditioning (Figure 4A). As it has been proposed that formation of a dimer is crucial for NO production by NOS, we evaluated the levels of eNOS uncoupling in HUVECs submitted to normoxia, hypoxia or hypoxic-preconditioning using low-temperature-SDS-PAGE. Figure 4B shows no difference in eNOS expression and in monomer formation in the 3 experimental conditions.

Although total eNOS expression was not affected by the different protocols, eNOS activating phosphorylation on serine 1177²⁷ was significantly increased in arteries from mice exposed to hypoxic preconditioning before the sustained hypoxia but remained unmodified in arteries from mice exposed to hypoxia alone (Figure 4C). Similar results were obtained in HUVEC samples (not shown). Interestingly, in arteries from TRPV4 KO mice, hypoxic preconditioning had the opposite effect—eNOS phosphorylation on serine 1177 was significantly inhibited (Figure 4D).

Hypoxia-Reoxygenation and Hypoxic Preconditioning Stimulate the Membrane Colocalization of Cav-1 and TRPV4

In our previous work, we evidenced an enrichment of TRPV4 in the caveolar low-density fractions, as well as a direct interaction between TRPV4 and cav-1.¹⁷ Thus, we verified the

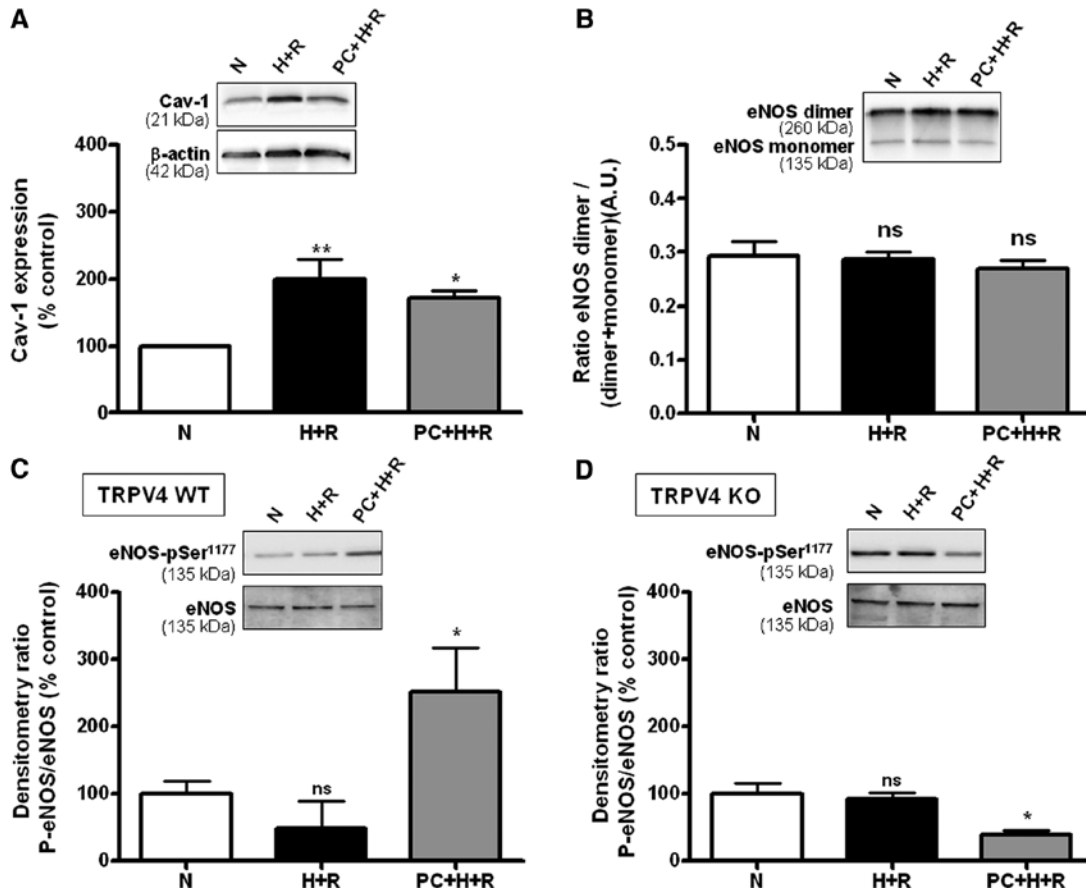


Figure 4. Effect of hypoxia-reoxygenation and hypoxic preconditioning on caveolin-1 (cav-1) expression, endothelial nitric oxide synthase (eNOS) uncoupling and eNOS phosphorylation (serine 1177). **A**, Immunoblotting analysis for cav-1 expression in human umbilical vascular endothelial cells (HUVECs) previously exposed to hypoxia-reoxygenation (H+R), hypoxic preconditioning preceding hypoxia-reoxygenation (PC+H+R), or kept in normoxia (N) (n=8). β -actin was used as loading control. **B**, Representative eNOS immunoblotting signals from nondenatured HUVEC lysates previously exposed to N, H+R, or PC+H+R with densitometric analysis of eNOS monomer/dimer, quantified from 4 independent preparations. **C** and **D**, Representative phospho-eNOS (P-eNOS) and eNOS immunoblotting signals from transient receptor potential cation channels V4 wild-type (WT) and knockout mouse aortas previously exposed to N, H+R, or PC+H+R. Densitometric analysis quantified from 5 independent preparations. ns indicates not significant.

impact of hypoxia-reoxygenation and preconditioning protocols on cav-1 and TRPV4 location. Immunostaining TRPV4 confirmed its increase under both conditions. Furthermore, both hypoxia and hypoxic preconditioning induced the translocation of TRPV4 channels to caveolin-enriched domains at the cell membrane as attested by the yellow dots in Figure 5.

Discussion

The current study provides new insights on the mechanisms underlying the response to hypoxic preconditioning in the vasculature. We found, indeed, that in conditions mimicking some aspects of ischemia and reperfusion, NO-mediated relaxation is reduced, whereas EDH(F)-mediated relaxation is upregulated. Preconditioning, however, preserves vascular function by restoring the NO-mediated relaxation and further improving the EDH(F)-mediated response. These data were obtained from vessels preconditioned *in vivo* by exposing mice to 3 short cycles of hypoxia-reoxygenation, making our observations particularly relevant in the understanding of this multivariable phenomenon. In particular, we found that *in vivo* gap junction uncoupling by carbenoxolone completely inhibited the EDH(F) pathway and significantly reduced the protection afforded by

preconditioning for the concomitant NO-mediated relaxation. Using cultured endothelial cells and mice aortas to dissect the possible underlying mechanisms, we demonstrated that TRPV4 channels, Cx40 and Cx43, directly participate in the vascular protection offered by hypoxic preconditioning, reinforcing the activation of the NO-dependent pathway.

The role of Cx43 as a selective mediator of pre- and post-conditioning was previously reported in cardiac myocytes.²⁸ Preconditioning was shown to cause a decrease in Cx43 dephosphorylation and translocation, thereby, influencing the rate of electrical uncoupling in the myocardium.²⁹ In the brain also, preconditioning against ischemic injury can be achieved by a diminished Cx43 degradation associated with the opening of connexin hemichannels and the final release of the neuroprotective ATP/adenosine.³⁰ In the current study that focuses on vascular tissue, we found that preconditioning (and not hypoxia-reoxygenation) led to an increase in both total expression of Cx40 and Cx43 and importantly also in the junction conductance as determined by the diffusion of a gap junction permeable tracker (see Figure 2C). These results suggest that modulation of gap junction coupling participates in the vessel relaxation improvement observed in response to preconditioning (Figure 1).

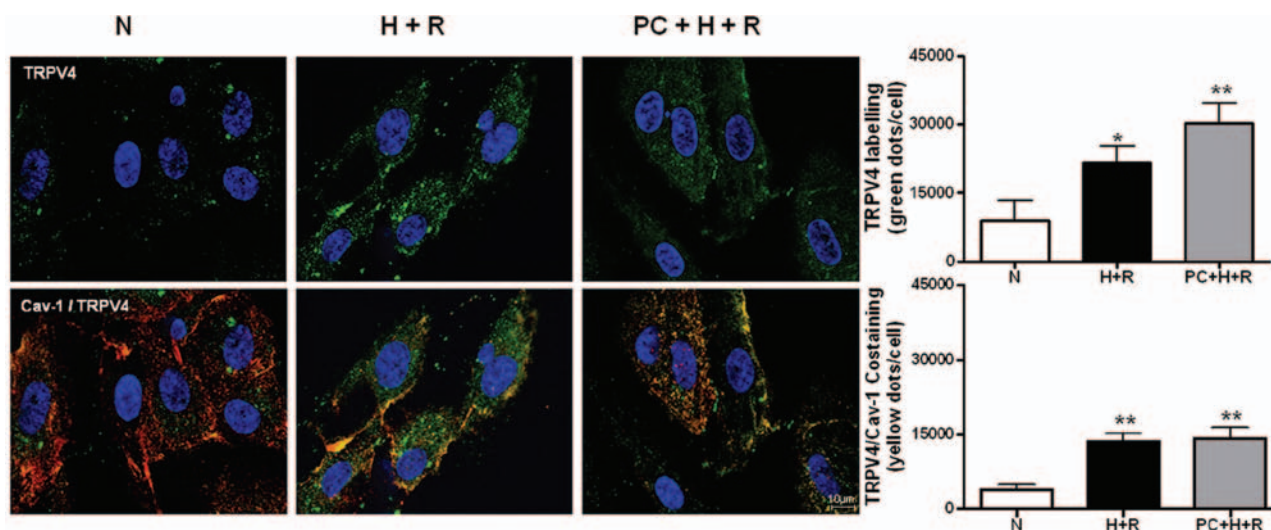


Figure 5. Effect of hypoxia-reoxygenation and hypoxic preconditioning on caveolin-1 (Cav-1) and transient receptor potential cation channels V4 wild-type (TRPV4) colocalization. Left, Representative immunocytochemistry images of Cav-1 (red) and TRPV4 (green) labeling in human umbilical vascular endothelial cells (HUVECs) previously exposed to hypoxia-reoxygenation (H+R), hypoxic preconditioning preceding hypoxia-reoxygenation (PC+H+R), or kept in normoxia (N). Colocalization is visualized as yellow dots, nuclei are DAPI stained (blue) (magnification $\times 63$ oil immersion objective lenses). Right, Quantitative analysis expressed as number of dots per cell from randomly selected images (>10) for each condition from 3 independent experiments.

Another key finding arising from the dose–response vaso-relaxation curves presented in Figure 1 was that prolonged hypoxia (followed by reoxygenation) itself stimulates the EDH(F) pathway, whereas it impairs the activation of eNOS. Using cultured endothelial cells, we identified the involvement of Ca^{2+} entry through TRPV4 channels shown to be a major mediator of the EDH(F) signaling cascade.^{17,31,32} Although the implication of the connexins could be exclusively related to the initial preconditioning challenge, the increased expression and activity of TRPV4 was favored by the hypoxic insult independently of the preconditioning protocol. Ischemia-reperfusion is known to be associated with an increased Ca^{2+} response in endothelial cells upon agonist stimulation, which contributes to the potentiated EDH(F)-mediated dilations.³³ We and others have shown that endothelial TRPV4 channels participate in both the NO and EDH(F)-mediated relaxation.^{17,34} Similarly, the epoxyeicosatrienoic acids compounds proposed to act as endothelium–derived hyperpolarizing factors, particularly in response to hypoxia-induced cytochrome P450 2C9 expression,³⁵ stimulate $[\text{Ca}^{2+}]_i$ increase in endothelial and smooth muscle cells through TRPV4 channel activation.³⁶ The above data support the induction of TRPV4 as a mediator of EDH(F) in response to hypoxia. Despite increase in TRPV4 expression under both hypoxic and preconditioning conditions, its deficiency only downsizes the increase of EDH(F)-mediated relaxation associated with hypoxia. Under preconditioning, the EDH(F) response is still significantly increased in TRPV4 KO mice (Figure 3D). Other vessel wall transient receptor potentials channels (eg, transient receptor potential channel [TRPC] 4) might be upregulated in these mice to compensate for TRPV4 deficiency^{37,38} and could account for this observation.

It is important to note that structural caveolar component cav-1 was found to be increased in membrane fractions after hypoxia-reoxygenation (independent of preconditioning) (Figure 4A). We previously documented that TRPV4 interacts with

cav-1,¹⁷ and we along with others have previously reported that connexins colocalize and interact with cav-1 in different cell types, including endothelial cells.^{17,39,40} With cav-1 regulating eNOS signaling,⁴¹ caveolae appears to be the key compartment for vascular function regulation. Indeed, cav-1/eNOS interaction results in steric inhibition of eNOS. Inhibition is reversed by calmodulin binding upon agonist–stimulated intracellular calcium increase, thus, liberating all downstream signaling involving various G protein coupled receptors.²⁷ At the same time, cav-1 favors calcium entry by a functional TRPV4 membrane targeting and ensures proper gap junction coupling by transporting newly formed connexins to the plasma membrane.^{17,39} How things may work in hypoxic and hypoxic preconditioning situations is illustrated in Figure 6. We evidenced that in hypoxic conditions (Figure 6A), vasodilation is preserved by an upregulation of EDH(F)-mediated relaxation requiring the increase of TRPV4 expression and functionality, which is interestingly associated with an increase of cav-1 at membrane locations. Under these conditions, NO-mediated relaxation is impaired partly because of enhanced caveolin suppressive interactions and because of free oxygen radical generation and impaired NO bioavailability. Negative regulation of eNOS might also occur through Cx37 interaction⁴² as some of our preliminary results suggested that Cx37 expression could be modulated in hypoxic conditions (not shown). Preconditioning protocol (Figure 6B) restores the NO-mediated pathway through a reduction of caveolin interactions. Moreover, preconditioning involves Cx40 and Cx43 increase, as well as a more efficient gap junction coupling. A recent work by Alonso et al⁴³ evidenced that Cx40 is necessary for proper eNOS expression and function; thus, Cx40 increase might eventually upregulate NO production in preconditioning. In addition, other reported interactions between eNOS and connexins⁴⁴ may account for our observations. Interestingly, we could evidence that TRPV4

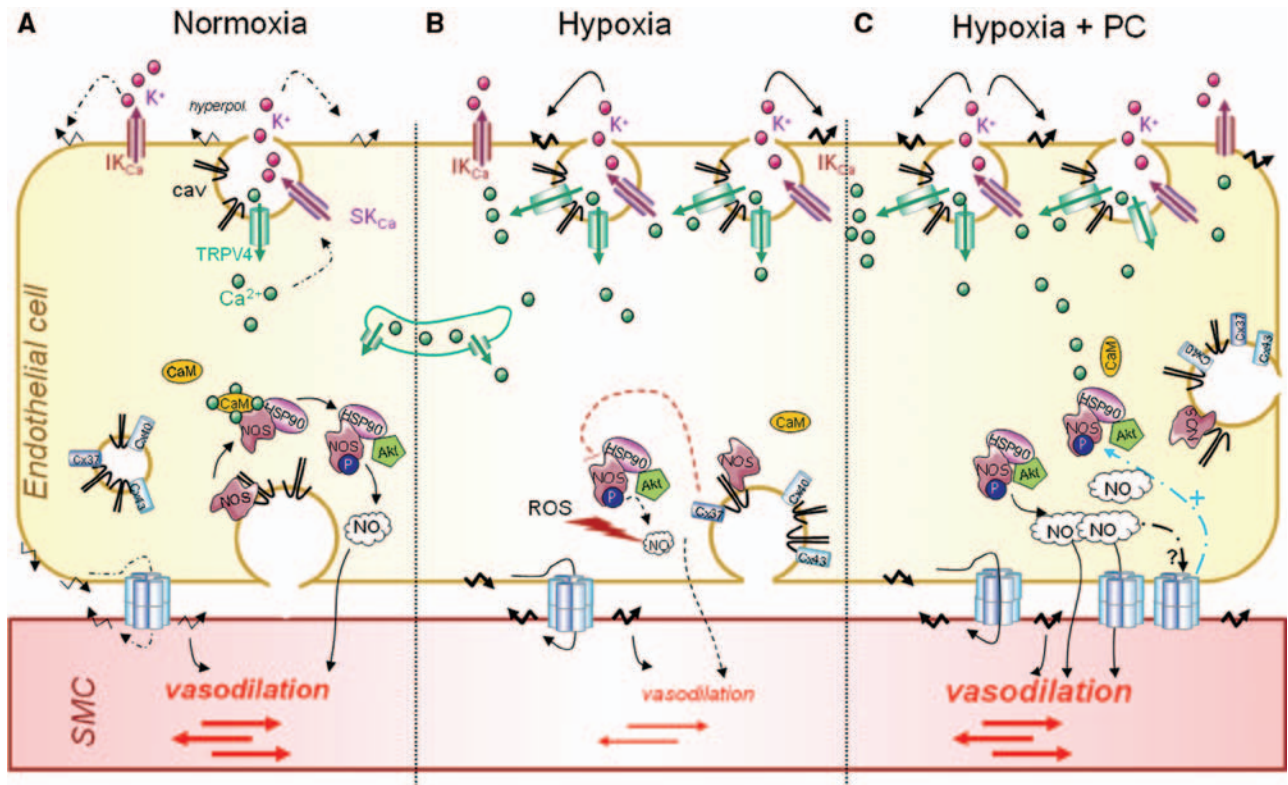


Figure 6. Involvement of transient receptor potential cation channels V4 (TRPV4), connexins, and their caveolar location in the control of vasomotor tone under ischemic and ischemic preconditioning. **A.** Normoxic conditions, caveolin-1/endothelial nitric oxide synthase (eNOS) interaction results in steric inhibition of eNOS. Upon agonist activation, an increase in intracellular calcium results in calcium-calmodulin binding, which can displace caveolin and reverse its inhibitory interaction with eNOS. At the same time, caveolin-1 favors endothelium-derived hyperpolarization EDH(F) signaling increasing calcium entry by a proper TRPV4 targeting and gap junction coupling by transporting newly formed connexins to the plasma membrane. **B.** Hypoxic conditions, NO production by eNOS is impaired. However, vasorelaxation is preserved by an upregulation of EDH(F)-mediated relaxation requiring the increase of TRPV4. **C.** Preconditioning protocol before prolonged hypoxia restores the NO-mediated pathway, which involves connexin (Cx) 40 and Cx43 protein expression increase and better gap junction coupling. SMC indicates smooth muscle cell; cav, caveolin; ROS, reactive oxygen species; hyperpol, hyperpolarization; HSP90, heat shock protein 90; CaM, calmodulin; P, phosphate.

is also involved in nitric oxide synthase regulation as phosphorylation on eNOS serine 1177 was increased upon preconditioning in presence of TRPV4 and inhibited in its absence, which corroborates with the recent findings of Adapala et al.⁴⁵ Conversely, TRPV4 might be regulated by the NO pathway as it has been shown in lung microvascular endothelial cells that cGMP is a critical regulator of TRPV4.⁴⁶ Notably, TRPV4 colocalizes and heteromerizes with transient receptor potential channel (TRPC) 1, which is a constituent of the endothelial caveolar signalplex/channelosome together with store-operated channels.⁴⁷ It is advisable also to follow up the transient receptor potential channel 1–TRPV4 interaction.

In summary, our work provides evidence on how TRPV4 and connexins might participate in the preservation of vasodilation under hypoxic conditions and restore the NO-mediated pathway in hypoxic preconditioning. Indeed, an increase in expression and activity of TRPV4 channels associated with a higher concentration of caveolae at the membrane probably potentiate the EDH(F) response under hypoxia. Promotion of intercellular coupling through gap junctions is the other necessary pathway to generate the vascular protective effect of preconditioning. Unexpectedly, pointing out caveolae as a common signaling platform, our results further suggest an intimate relationship between NO and EDH(F) pathways.

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Disclosures

None.

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