Objective—We investigated the impact of hypoxia-reoxygenation on endothelial relaxation and aimed to clarify the role of transient receptor potential cation channels V4 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.

Key Words: hypoxic preconditioning ■ gap junctions ■ transient receptor potential cation channels V4 ■ NO ■ endothelium-derived hyperpolarization

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

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vasodilation after ischemia-reperfusion, and the protective role of EDH(F) in ischemic preconditioning remains elusive.\textsuperscript{12–14} Some key mediators of EDH(F) signaling are nowadays clearly identified. Accordingly, EDH(F) is triggered by an elevation of cytoplasmic Ca\textsuperscript{2+} concentration in endothelial cells ([(Ca\textsuperscript{2+})])\textsuperscript{15} and the final opening of Ca\textsuperscript{2+}-activated potassium channels (K\textsubscript{Ca}) expressed either on endothelium (most likely IK\textsubscript{Ca} and SK\textsubscript{Ca} channels) or on smooth muscle cells (most likely BK\textsubscript{Ca} channels).\textsuperscript{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 their genetic deletion affected both the NO and EDH(F) components of the relaxation to muscarinic cholinergic stimulation.\textsuperscript{17} Also, in many resistance vessels, vascular gap junction integrity is a requisite for vascular smooth muscle cell hyperpolarization to occur.\textsuperscript{18,19} We have demonstrated that the EDH(F)-mediated relaxation was totally absent\textsuperscript{17} 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 placed in a modular incubator chamber and exposed or not, for 16 hours, to 13% O\textsubscript{2} and 5% CO\textsubscript{2}.\textsuperscript{20} For preconditioning, prolonged hypoxia was preceded by 3 periods of 15-minute hypoxia (6% O\textsubscript{2} and 5% CO\textsubscript{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 µL 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.\textsuperscript{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\textsubscript{2}) or under hypoxia (1% O\textsubscript{2}, 5% CO\textsubscript{2}, and 94% N\textsubscript{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.\textsuperscript{21} Nondenaturating 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, phospho-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 described\textsuperscript{22} and validated,\textsuperscript{23,24} was used. Briefly, confluent HUVECs were labeled with 1 µmol/L calcine-AM. Excess (extracellular) calcine 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 µmol/L membrane-bound PKH26. The 2 populations were exposed to normoxia (control) or hypoxia preceded or not by preconditioning. Then, PKH26-labeled HUVECs were trypsinized and added to the calcine-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 stained indicated calcine dye transfer into PKH26-labeled cells.

Measurement of [Ca\textsuperscript{2+}], in HUVECs

[Ca\textsuperscript{2+}], was determined with the fluorescent Ca\textsuperscript{2+} indicator Fura-2 as previously described.\textsuperscript{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–donkey anti-goat IgG and fluorescein isothiocyanate–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, ×63 oil immersion objective lenses, and analyzed with AxioVision software.

Statistical Analysis

All results are expressed as mean±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. Unmarqued or ns, P>0.05; *P<0.05; **P<0.01; ***P<0.001. For Figure 2: *P<0.05; **P<0.01 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 solution (to exclude any EDH(F)) and exposed to ace-tylcholine (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-preconstricted arteries (6 µmol/L) were exposed to acetylcholine (0.01–1 µmol/L) in presence of a nitric oxide synthase inhibitor (NG-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. 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 exposure 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 we evaluated the expression and activity of these channels in endothelial cells exposed to hypoxia-reoxygenation and preconditioning. As shown in Figure 3A, hypoxia-reoxygenation preceded or not by 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 Ca\(^{2+}\) turnover, the effect of 4\(\alpha\)PDD (10 µmol/L), a highly specific activator of TRPV4 channels on \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) signal increase evoked by 4\(\alpha\)PDD 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 \([\text{Ca}^{2+}]\) concentrations amounted to 50±8 nmol/L in normoxia, 116±15 nmol/L in hypoxia, and 130±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 EDHF-mediated dilations. Consistent with previous data, NO- and EDHF-dependent relaxation were markedly depressed in normoxic TRPV4 KO mice arteries and hypoxia-reoxygenation preceded or not by hypoxic preconditioning had no 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 EDHF-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 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-1 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).

eNOS alteration is also proposed in various studies as to arise from eNOS uncoupling. We, thus, determined the levels of eNOS monomer and dimer in HUVECs submitted to normoxia, hypoxia, or hypoxic preconditioning using LT-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 1177 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. Thus, we verified the 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 postconditioning was previously reported in cardiac myocytes. Preconditioning was shown to cause a decrease in Cx43 dephosphorylation and translocation, thereby, influencing the rate of electrical uncoupling in the myocardium. 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. 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).
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 EETs, compounds proposed to act as endothelium–derived hyperpolarizing factors, particularly in response to hypoxia-induced CYP2C9 expression,\(^ {35}\) stimulate [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 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.\(^ {17,30,39}\) With cav-1 regulating eNOS signaling,\(^ {31}\) 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.\(^ {17,32}\) 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 interaction 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 al\(^ {43}\) 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 connexins\(^ {44}\) may account for our observations. Interestingly, we could evidence that TRPV4...
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 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.

**References**


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