Abrupt reoxygenation following hypoxia reduces electrical coupling between endothelial cells of wild-type but not connexin40 null mice in oxidant- and PKA-dependent manner

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ABSTRACT

Although electrical coupling along the arteriolar endothelium is central in arteriolar conducted response and in control of vascular resistance, little is known about the pathophysiological effect of hypoxia and reoxygenation (H/R) on this coupling. We examined this effect in a monolayer of cultured microvascular endothelial cells (ECs) derived from wild-type (WT) or connexin (Cx)40−/− mice (Cx40 is a key gap junction protein in ECs). To assess electrical coupling, we used a current injection technique and Bessel function model to compute the monolayer intercellular resistance. Hypoxia (0.1% O2, 1 h) followed by abrupt reoxygenation (5–90 min) reduced coupling (i.e., increased resistance) in WT but not in Cx40−/− monolayer. H/R increased superoxide production and reduced protein kinase A (PKA) activity in both monolayers. Activation of PKA by 8-bromo-cAMP prevented the reduction in coupling. Preloading of the WT monolayer with the antioxidant ascorbate prevented reductions in both PKA activity and cell coupling. Inhibition of PKA with 6-22 amide during normoxia mimicked the reduction in coupling. Finally, hypoxia followed by slow reoxygenation mimicked the reduction in superoxide level, PKA activity, or coupling. Using intravital microscopy, we assessed the physiological relevance of these findings in terms of KCI-induced conducted vasoconstriction in arterioles of WT mouse cremaster muscle in vivo. Ischemia (1 h) followed by abrupt reperfusion (15–30 min) reduced conduction. 8-bromo-cAMP prevented this reduction, while 6-22 amide mimicked this reduction in control nonischemic arterioles. We propose that abrupt reoxygenation reduces interendothelial electrical coupling via oxidant- and PKA-dependent signaling that targets Cx40. We suggest that this mechanism contributes to compromised arteriolar function after H/R.

Key words: vascular resistance • Bessel function model • cremaster muscle
Vascular cell coupling has been studied extensively at the arteriolar level where it is thought to coordinate blood perfusion in the tissue. Coupling of cells within the arteriole has been characterized in terms of the arteriolar conducted response where locally elicited dilation/constriction spreads quickly along ~2–3 mm length of the arteriole (1). On the basis of direct electrophysiological measurement within the arterial wall, it has been demonstrated that the principal pathway of the conducted response is conduction of hyper/depolarizing electrical currents along the endothelial cell (EC) layer of the blood vessel (2, 3). Electrical coupling along endothelial cells is achieved through gap junctions (GJs) (4).

Arterioles are exposed to hypoxia/reoxygenation (H/R) during pathophysiological events involving ischemia/reperfusion. It is not known how H/R affects coupling within the arteriolar wall and the attendant arteriolar function. Because the principal electrical pathway of arteriolar conduction occurs along the endothelium, we recently used an in vitro model of microvascular EC monolayer to study the effect of H/R on electrical coupling in the vasculature. We have recently showed in rat EC monolayers that a short-term H/R (i.e., 1 h hypoxia followed by 4–100 min reoxygenation) reduces electrical coupling without causing cell damage (5). However, in these experiments, we did not identify the target of the H/R-induced signaling, that is, which GJ protein may be responsible for this reduction.

Among vascular connexins (Cx) known to be expressed in ECs (i.e., Cx37, Cx40, Cx43), Cx40 appears to play the central role in the arterial conducted response (6, 7). This is supported by evidence showing that knockout of Cx40 results in an impaired conduction of vasodilation along arterioles in mouse cremaster muscle (6, 7). Because of this and the fact that Cx40 gap junctions have high single-channel conductance (8), Cx40 could dictate the baseline intercellular macroscopic conductance and be responsible for the reduced coupling after H/R.

It is well established that H/R results in a marked increase in reactive oxygen species (ROS) (9). This mainly occurs through the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase (during hypoxia), and the subsequent reduction of molecular O2 to superoxide during reoxygenation (10). The increase in ROS is thought to contribute to vascular dysfunction involving numerous signaling pathways, including inactivation of protein kinase A (PKA) (11, 12). Van Rijen and co-workers have shown that Cx40 GJ macroscopic conductance could be modulated by intracellular cAMP level (and thus by PKA activity).

Accordingly, in the present study, we hypothesized that reduction in interendothelial coupling following short-term H/R is due to modulation of Cx40 function. We further hypothesized that PKA and H/R-generated ROS play key roles in the reduction in coupling. We show for the first time that H/R-reduced PKA activity and attendant increase in ROS level could target Cx40 and reduce interendothelial cell coupling.

**MATERIALS AND METHODS**

**Reagents**

The 6-22 amide, 8-bromo cAMP, spermine HCl, and anti β-actin antibody kit were purchased from Calbiochem (La Jolla, CA). Heparin was purchased from Leo Laboratories (Ajax, ON, Canada). Fetal bovine serum (FBS), dialyzed FBS, antibiotic-mycotic solution, L-glutamine,
trypsin-EDTA were purchased from Gibco (Mississauga, ON, Canada). DMEM/F12, DMSO (DMSO), Nitro Blue Tetrazolium (NBT), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), GS-I lectin, L-ascorbate, 5(6)-carboxyfluorescein diacetate, cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). Endothelial growth supplement was purchased from Collaborative Research (Bedford, MA). Magnetic beads and the magnetic particle concentrator were purchased from Dynal (Lake Success, NY). Mouse monoclonal anti-Cx43 antibody was purchased from Transduction Laboratories (Bio/Can Scientific, Mississauga, ON, Canada). A peroxidase-labeled anti-mouse IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Cx40 and anti-Cx37 antibodies were purchased from Chemicon (Temecula, CA) and Alpha Diagnostics (San Antonio, TX), respectively. The anti-Cx40 antibody does not cross-react with Cx37 or Cx43, while the anti-Cx37 antibody does not cross-react with Cx40 or Cx43 (13). Peroxidase-labeled anti-rabbit IgG was purchased from Cell Signaling Technology (Beverly, MA). The enhanced chemiluminescence kit was purchased from LUMIGLO, KPL Laboratories (Gaithersburg, MD).

Isolation and culture of mouse microvascular endothelial cells

Experiments were approved by the Council on Animal Care at the University of Western Ontario. We used male wild-type (WT) and Cx40 deficient (Cx40−/−) C57BL/6 mice. Several Cx40−/− male mice were kindly provided by Dr. David Paul (Harvard University, Boston, MA) (14). Additional male Cx40−/− mice were produced by crossing Cx40−/− males with WT C57BL/6 females and by breeding the heterozygous offspring. Using a standard genotyping procedure, we confirmed the absence of Cx40 mRNA in Cx40−/− mice. Isolation of mouse microvascular endothelial cells (MMECs) was based on a procedure described by us (15). Briefly, the hindlimb muscle of mice was excised, minced, and digested in an enzyme solution. Digest was filtered through a nylon mesh, and cells were collected and washed in DMEM/F12. Cells were grown to confluence and then subjected to purification by immunoseparation using GS-I lectin-coated beads. Pure MMEC were then cultured in maintenance medium containing DMEM/F12, FBS (10%), endothelial growth supplement (100 μl/ml), heparin (5 U/ml), L-glutamine (0.1 μg/ml), and antibiotic-mycotic solution (10 μl/ml) in standard incubator conditions. Cells were used between passages 6 and 13. Endothelial phenotype was determined by the presence of von Willebrandt factor VIII and GS-I lectin antigens as detailed by us (16).

Exposure of cells to hypoxia, and to abrupt or slow reoxygenation

Two hours before all experiments, the maintenance medium was replaced by a dialyzed serum medium (DSM) for the duration of the experiment (i.e., FBS was replaced by 5% dialyzed serum (17)). Hypoxic DSM was prepared by bubbling with 100% N2 for 5 min or by placing DSM into hypoxic incubator overnight (5% CO2, 0.1% O2 and 94.9% N2 at 37°C). To begin hypoxia, cells were covered by ~2 mm thick layer of hypoxic DSM and placed into hypoxic incubator. At the end of hypoxia (i.e., 1 h for all experiments), cells were subjected either to abrupt or slow reoxygenation. For abrupt reoxygenation, hypoxic DSM was replaced by normoxic DSM and cells were placed into a standard normoxic incubator for a specific time period. Normoxic DSM was either fresh (i.e., DSM was kept in normoxic incubator for at least 1 h) or “conditioned” by cells during hypoxia (i.e., hypoxic DSM collected from separate cells after 1 h hypoxia was re-equilibrated with oxygen in normoxic incubator for at least 1 h). For slow reoxygenation, cells were left covered by the hypoxic medium (unstirred) and were placed into the normoxic
incubator. Here, room air oxygen had to diffuse to the cells through the 2-mm layer (i.e., reoxygenation was slower than that of the abrupt reoxygenation procedure). In this paper, "H/R\textsubscript{o}" and "H/R\textsubscript{ac}" refer to hypoxia followed by abrupt reoxygenation with fresh or conditioned DSM, respectively, while "H/R\textsubscript{s}" refers to hypoxia followed by slow reoxygenation. Our control cells (with or without treatments described below) were subjected to the same experimental steps as the H/R cells, except that normoxic DSM and standard normoxic incubator were used instead of hypoxic DSM and hypoxic incubator, respectively.

**Western blot analysis**

Proteins were resolved on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with nonfat dry milk. For Cx37 and Cx40 immunoblotting, 50 μg/well were loaded and membranes were incubated with anti-Cx37 or anti-Cx40 antibody (1:500) overnight at 4°C. Membranes were then washed and further incubated with peroxidase-labeled anti-rabbit IgG antibody (1:2000, 1 h at room temperature). For Cx43 immunoblotting, 10 μg/well were loaded, and membranes were incubated with anti-Cx43 antibody (1:1000) for 1 h at room temperature. Membranes were then washed and further incubated with peroxidase-labeled anti-mouse IgG antibody (1:2000, 1 h at room temperature). All blots were then washed, and banding was visualized using an enhanced chemiluminescence kit with Kodak BIOMAX MS imaging film. For protein loading control, blots were stripped and reprobed for β-actin using anti β-actin antibody (1:5000, 1 h at room temperature), washed, and further probed with peroxidase-labeled anti-mouse IgM antibody (1:2000) and subsequently visualized.

**Electrophysiology**

To assess coupling, we determined intercellular resistance (i.e., inverse measure of coupling) based on electrophysiological approach described by us (15). Briefly, cells grown in monolayers (on glass coverslips) were injected with 4 or 5 hyperpolarizing pulses (25 nA, 100-ms pulse width). The resulting deflection from the resting membrane potential (\(E_m\)) in cells was recorded at various distances along the monolayer (i.e., distances were measured between the injecting and recording electrodes) as exemplified in Fig. 1. For increasing distances from 50 to 400 μm, \(E_m\) deflections ranged 2–30 mV. On the basis of the recordings, the monolayer thickness of 1.9 μm (5) (short-term H/R does not affect the thickness (5)), and a Bessel function model, the intercellular resistance (\(r_i\)), transmembrane resistivity (\(R_m\)), and space constant (\(\lambda\)) were determined. Because electrophysiology was done in room air (cell chamber heated to 37°C), cells subjected to abrupt reoxygenation were covered by normoxic DSM, including 25 mM HEPES (i.e., to maintain pH at 7.3). For slow reoxygenation experiments, HEPES was also included in the hypoxic DSM (i.e., hypoxic incubator was made free of CO\(_2\)).

Our previous work in rat microvascular ECs showed variability in baseline intercellular resistance (e.g., reflecting experiment-to-experiment variability). To control for this variability, for each experimental day, we simultaneously prepared the appropriate number of monolayer-covered glass coverslips, which were then randomly assigned to normoxia or H/R treatment groups. Within these groups, coverslips were also randomly assigned to pretreatment subgroups, including 8-bromo cAMP (1 mM, cell-permeable cAMP analog, PKA activator), 6-22 amide (10 nM, competitive inhibitor of PKA), the antioxidant ascorbate (200 μM), spermine (2 mM), or
cycloheximide (10 μg/ml). Concentrations of agents used were based on the manufacturer's recommended concentrations, published reports, and on our preliminary experiments.

Fluorescence recovery after photobleaching

In the present study, we also used an alternative approach to assess intercellular coupling based on fluorescence recovery after photobleaching (FRAP) (18, 19). Briefly, wild-type cells grown in monolayers were exposed to normoxia or hypoxia for 1 h, and then exposed to fresh normoxic DSM with 10 μg/ml 5(6)-carboxyfluorescein diacetate dye at 37°C for 20 min. The cells were then washed twice with DMEM/F12 (to remove excess dye) and placed on a special stage of a Zeiss LSM 510 META confocal microscope (the stage permitted maintenance of cells in air plus 5% CO₂ atmosphere at 37°C). Within 5 min after this placement, the baseline fluorescence of the dye was recorded in a randomly selected group of 4 or 5 cells. These cells were then photobleached with a 488-nm argon laser beam to ~50% of their original intensity, and the recovery of fluorescence within the cells was recorded every 4 s for the total of 5 min. Figure 1B exemplifies the fluorescence intensity data obtained throughout the 5-min period. All recordings were done with identical settings of the confocal microscope.

Intracellular PKA and reactive oxygen species

PKA activity was determined using an enzyme-linked immunosorbent assay-based, nonradioactive kit from Calbiochem (20). We used the NBT assay to assess superoxide production before and after H/R (21). Briefly, cells were grown to confluence and then treated with NBT (10 mg/ml) in DSM for 2 h before and during, experiments. Cells were then washed with phosphate-buffered saline and then quickly rinsed with NaOH (2 M). DMSO was added to each well to dissolve NBT; its absorbance was determined at 654 nm.

For both assays, we plated cells so that consistent confluency among dishes was reached at the time of the experiment. This consistency was verified by measuring the protein amount (Bio-Rad, DC Protein Assay) in some of these dishes selected at random (outcome: no more than 5% difference in protein was seen between dishes).

Relevance of in vitro experiments to an in vivo model of vascular cell coupling

Because present in vitro experiments in MMECs could be challenged in terms of their physiological relevance to vascular cell coupling in vivo, we aimed to examine the effect of ischemia/reperfusion (I/R) on the arteriolar conducted response (i.e., H/R is a key component of the complex vascular response to I/R). To this end, we used arterioles in the mouse cremaster muscle as detailed by us (22). Briefly, male WT C57BL/6 mice were anesthetized with ketamine/xylazine (ip injection), and the muscle sac was isolated from the scrotum and placed on a glass platform. A longitudinal incision was made in the ventral surface of the muscle, and the muscle was gently spread over the platform and irrigated with physiological saline solution (PSS), 33–34°C, pH 7.4, composed of (in mM) 131.9 NaCl, 4.7 KCl, 2.2 CaCl₂, 1.2 MgSO₄, and 20.0 NaHCO₃ bubbled continuously with 5% CO₂/95% N₂ gas. The muscle was epi-illuminated and visualized with an intravital microscope, and the resultant field of view (0.65 × 0.48 mm) was video recorded. The vasodilative ability of arterioles was tested in terms of the effect of the
NO donor S-nitroso-N-acetyl-penicillamine (SNAP; 50 μM in PSS) on preconstricted arterioles (i.e., after ~5 min superfusion with PSS bubbled with 10% O₂+5% CO₂+85% N₂).

Glass micropipettes were backfilled with 3.0 M KCl in PSS and connected to a Picospritzer II (General Valve). To initiate the conducted response, unbranched arterioles (~50 μm in diameter, ~2 mm long, 1A or 2A branching order, one arteriole per mouse) at the muscle surface were stimulated by pressure ejected KCl onto the arteriole (average pulse duration ~140 ms at pressure 50 psi). The pulse duration was adjusted to yield a local constriction of ~50%. Subsequent stimulations were repeated at the same local site using the same pulse duration. During stimulations, arterioles were superfused with PSS at a rate of 1 ml/min such that the KCl puff was carried in a direction away from the 500-μm upstream site. We simultaneously video recorded diameter changes occurring at the local and 500-μm upstream sites. The luminal arteriolar diameter at both sites was measured from the video screen at three time points: pre-KCl (D_{local,pre} and D_{500,pre}), maximal constriction (i.e., minimal diameter, D_{local,min} and D_{500,min}) occurring at 3–4 s post-KCl (22), and at 30 s post-KCl. The relative diameter changes for the local and upstream sites were ΔD_{local} (%) = 100% × (D_{local,pre}−D_{local,min})/D_{local,pre} and ΔD_{500} (%) = 100% × (D_{500,pre}−D_{500,min})/D_{500,pre}, respectively. The communication ratio, CR_{500} = ΔD_{500}(%) / ΔD_{local}(%), was used as an index of the arteriolar conducted response (22).

After 30 min postsurgical stabilization, the control baseline vasoconstrictor-conducted response was determined (i.e., at the −120 min time point). The cremaster preparation was then irrigated (less than 2 ml) either with PSS, 8-bromo cAMP (10 mM in PSS) or 6-22 amide (100 nM in PSS). A plastic cover was placed over the preparation for 1 h and then KCl-induced conducted response was again evaluated (i.e., at −60 min). Next, the muscle pedestal was cross-clamped to achieve a complete 1 h of ischemia; the release of clamp marked the beginning of abrupt reperfusion (0 min). Conducted responses were evaluated at 15, 30, and 60 min after the beginning of reperfusion.

Statistics

Data were expressed as means ± SE (n indicates the number of monolayers used per treatment group, unless otherwise stated) and were based on MMECs isolated from at least three different mice. Data were analyzed by ANOVA followed by Tukey’s multiple comparisons test or Dunnett’s test, as indicated. Significance was assigned at P < 0.05.

RESULTS

Cell morphology and connexin expression

Confluent MMEC monolayers tested positive for von Willebrandt factor VIII expression and Griffonia simplicifolia lectin binding (data not shown), confirming the endothelial phenotype of our cells. Hypoxia (1 h) followed by abrupt reoxygenation (5–210 min) did not change the morphological appearance of the cells. H/R (30 min) and did not alter expression of Cx37, Cx40, and Cx43 in WT MMECs, as determined by immunoblot analysis (Fig. 2). In conjunction with these blots, we tested the sensitivity of Western blot technique to detect differences in connexin expression. To this end, the cells were treated with the protein synthesis inhibitor cycloheximide (10 μg/ml for 18 h), and the expression of Cx37, Cx40, and Cx43 was examined.
Using our blotting technique, cycloheximide was found to significantly reduce expression of all three connexins (data not shown). Our finding of unchanged connexin expression after H/R is consistent with the reported lack of effect of short-term H/R on Cx43 expression in rat microvascular ECs (5).

**Effect of H/R on coupling in wild-type and Cx40−/− MMEC**

H/R (5–90 min) did not alter the resting $E_m$ (control: $-16.2±0.4$ mV, $n=64$; H/R: $E_m=-15.2±0.3$ mV, $n=73$). Hypoxia followed by abrupt reoxygenation with fresh DSM increased intercellular resistance (i.e., reduced coupling) during the first 90 min; at 2 h of reoxygenation, resistance returned to baseline (Fig. 3A), indicating that reduced coupling was due to H/R-induced signaling rather than permanent cell injury. Abrupt reoxygenation with conditioned DSM resulted in the same increase in resistance as reoxygenation with fresh DSM (Fig. 4A, bars 2 and 3). We also used FRAP as an alternative method to assess intercellular coupling. Figure 3B demonstrates that H/R significantly reduced coupling as seen from H/R-induced reduction in fluorescence recovery after photobleaching.

Notably, Fig. 4A (bar 4) demonstrates that hypoxia followed by slow reoxygenation in wild-type MMECs did not alter $r_i$. Figure 4A also shows that H/R (5–40 min) in Cx40−/− MMECs did not affect $r_i$. To corroborate this finding, we used spermine (2 mM) in WT cells to inhibit Cx40 function. At this concentration, spermine was shown to specifically inhibit Cx40 channel conductance (23, 24). We found that 1 h treatment with spermine resulted in no change in $r_i$ after H/R (normoxia+spermine group: $1.34±0.07$ MΩ, H/R+spermine group: $1.24±0.12$ MΩ, $n=5$ per group). These results support the outcome of our experiments in Cx40−/− MMECs, demonstrating that Cx40 plays a pivotal role in the H/R-induced reduction in interendothelial electrical coupling.

**Involvement of PKA pathway and ROS**

Because it has been shown that Cx40 protein contains a consensus sequence for PKA and that PKA activation increases the Cx40 macroscopic gap junctional conductance (25), we tested for the involvement of PKA pathway in the H/R effect on $r_i$ in WT MMECs. Figure 4B addresses this involvement and shows that PKA activation by 8-bromo cAMP inhibited the effect of H/R on $r_i$, and that PKA inhibition with 6-22 amide in control cells close to the level seen after H/R. Figure 5 demonstrates that H/R reduced PKA activity and that treatments with 8-bromo cAMP/6-22 amide did elevate/reduce PKA activity in WT MMECs. Figure 6A (bars 4 and 5) shows that H/R-induced reduction in PKA activity was also seen in Cx40−/− cells. Interestingly, hypoxia followed by slow reoxygenation in wild-type cells did not alter PKA activity (Fig. 6A, bar 3).

To ascertain the role of ROS in H/R-induced increase in $r_i$ and a possible interaction of ROS with the PKA pathway, we measured ROS production in cells subjected to abrupt and to slow reoxygenation (i.e., procedures intended to generate different levels of ROS). Figure 6B confirms that, in wild-type cells, abrupt reoxygenation indeed increased ROS production, while slow reoxygenation did not (abrupt reoxygenation also increased ROS in Cx40−/− cells). Thus, in wild-type cells, increased ROS level after abrupt reoxygenation correlated with reduced PKA activity and increased $r_i$, while an unchanged level of ROS after slow reoxygenation was associated with
no changes in PKA activity and ri (Figs. 4–6). The role of ROS in H/Rα-induced reduction in PKA activity and in increased ri was underscored by our findings that preloading of MMEC with the antioxidant ascorbate prevented both the reduction in PKA activity (Fig. 6A, bars 6 and 7), as well as the H/Rα-increased ri (Fig. 7).

Baseline ri, and effect of H/R on transmembrane resistivity (Rm), and space constant (λ)

We observed that the baseline ri in normoxia varied experiment-to-experiment. Despite this variability, H/Rα (5–90 min) caused significant and consistent increases in ri in all experiments under control WT conditions. However, when considered on the basis of individual experiments, H/Rα-induced changes in Rm and λ were not always consistent, because of the low n value per experiment. To assess the overall effect of H/Rα on Rm and λ, we pooled all control and all H/Rα data. On the basis of these data, we found that H/Rα did not affect Rm (control: 11.69±1.27, n=24; H/Rα: 9.57±1.24 kΩ.cm², n=28), but it significantly decreased λ (control: 761±105; H/Rα: 305±11 μm). On the basis of the pooled data, ri increased significantly by ~100% (Control: 1.89±0.11; H/Rα: 3.80±0.20 MΩ).

Relevance of H/Rα-induced reduction in coupling in vitro to arteriolar conducted response

Figure 8 summarizes our findings on the local arteriolar responsiveness to KCl, and on KCl-initiated conducted response (i.e., CR500 values) in the mouse cremaster muscle. We found that the arteriolar ability to locally constrict was not altered by I/R or any of the treatments. However, the conducted response was reduced by I/R. This reduction manifested two features seen in our MMECs in vitro model. First, the reduction was temporary (i.e., observed up to 30 min of reperfusion) and, second, it was PKA-dependent. Similar to Fig. 4B, PKA activation by 8-bromo cAMP prevented the effect of I/R, while inhibition of PKA mimicked I/R in the preischemic arteriole (i.e., at –60 min), and prevented the recovery at 60 min of reperfusion.

DISCUSSION

The present study shows for the first time that 1 h hypoxia followed by abrupt but not slow reoxygenation reduces electrical coupling between WT mouse microvascular endothelial cells in vitro. This occurs via an oxidant- and PKA-dependent mechanism. Notably, abrupt reoxygenation does not reduce coupling in cells from Cx40−/− mice. Our data from WT mouse cremaster muscle arterioles underscore the physiological relevance of these findings, since 1 h ischemia followed by abrupt reperfusion reduced the arteriolar conducted response in vivo also PKA-dependently.

Our finding of H/Rα-increased ri in MMEC (~100% increase) is consistent with our previous study, in which comparable short-term H/Rα also increased ri in rat microvascular ECs (i.e., by 32%) (5), and with the present FRAP data showing a significantly lower fluorescence recovery after H/Rα (Fig. 3B). Our data are also consistent with the effect of long-term H/R (12–16 h hypoxia plus 1–2 h reoxygenation), which reduced coupling in both HUVEC (18, 26, 27) and rat cortical astrocytes (28). We recently found that hypoxia alone may not reduce electrical coupling in rat ECs (i.e., NaCN chemical hypoxia had no effect) (5). The present data (Fig. 4A) are consistent with this finding since no reduction in coupling was seen during the first 5 min of slow reoxygenation (i.e., when minimal oxygen reached the cells).
The H/Rα-reduced coupling most likely occurs during reoxygenation. During this phase, accumulated metabolites (e.g., hypoxanthine) are substrates for generation of large amount of ROS (29, 30). Figure 6B shows that hypoxia followed by abrupt reoxygenation was indeed associated with a significant increase in ROS, whereas slow reoxygenation caused no increase in ROS when compared with control. We believe that, in the case of slow reoxygenation, endogenous antioxidants scavenged the minimally produced ROS here, resulting in no detectable increase in ROS. ROS are most likely involved in the abrupt reoxygenation-induced reduction in coupling, since preloading of MMEC with the antioxidant ascorbate (i.e., a procedure reported to reduce intracellular ROS in ECs (31)) prevented the effect of abrupt reoxygenation on coupling (Fig. 7). These observations agree with the report that hydroxyl radicals play an important role in reduced coupling during the reoxygenation phase in HUVEC subjected to long-term H/R (32).

The mechanism of reduced electrical coupling following H/Rα is yet to be determined. Figures 4–6 indicate that ROS-mediated reduction in PKA may be a crucial signaling pathway here. Manipulation of ROS production by abrupt and slow reoxygenation (Fig. 6B) altered PKA activity (Fig. 6A, bars 1–3), while the antioxidant ascorbate prevented the H/Rα-induced reduction in PKA activity (Fig. 6A, bars 6 and 7). Consistent with these findings, Hastie and co-workers demonstrated that increase in oxidative stress reduces PKA activity in human umbilical vein endothelial cells (11), while superoxide inhibited activity of purified PKA from rabbit skeletal muscle (12). Further, it was shown that H/R-induced protein synthesis and cardiomyocyte hypertrophy are PKA-dependent (33).

In addition to the modulation of PKA activity, H/R has been shown to affect protein tyrosine kinase (PTK) and MAP kinase signaling (34, 35). We recently demonstrated that H/Rα increases phosphorylation of p44/42, while pretreatment with PTK and MAP kinase inhibitors prevents H/Rα-reduced in coupling in rat ECs (5). Because two structurally different inhibitors for both PTK and MAP kinases were used, it is unlikely that these inhibitors nonspecifically activated PKA to explain the protection against the H/Rα effect on coupling (Fig. 4B). Thus, it is possible that PTK, MAP kinase and PKA pathways (or possibly other mechanisms) are all involved in H/Rα-induced reduction in coupling.

Despite the apparent complexity of H/Rα-induced signaling leading to reduced coupling, the work of van Rijen and co-workers points to the possibility that reduction in PKA activity (Fig. 6A, bars 1 and 2) directly reduces coupling in our MMEC (25). They showed that, in communication-deficient cells subsequently transfected with Cx40, PKA activation by 8-bromo cAMP quickly increased (~50%) the Cx40 macroscopic conductance. Because Cx40 protein contains a consensus sequence for PKA and because 8-bromo cAMP increased phosphorylation of Cx40 (25), it is possible that PKA activation alone was responsible for the increase in Cx40 macroscopic conductance. Referring to Fig. 4A (bars 5 and 6), the effect of H/Rα was absent in Cx40−/− cells despite the presence of H/Rα-induced signaling (Fig. 6). We propose, therefore, that in our WT MMECs, H/Rα reduces Cx40 macroscopic conductance directly via reduced PKA activity.

At first glance, there is discrepancy between this proposed direct effect of PKA pathway on coupling and the complex involvement of PTK and MAP kinase pathways discussed above. To our knowledge, involvement of PTK or MAP kinase in Cx40 function is not known, and thus it is not clear whether these kinases cooperate with PKA to affect Cx40 gap junctional...
conductance. However, PTK or MAP kinase signaling was shown to affect tyrosine/serine phosphorylation of Cx43 and subsequently reduce coupling (17, 36). To reconcile the involvement of Cx40 and Cx43 in the H/R effect on coupling, it is possible that gap junctions consisting of mixture of connexins (e.g., Cx43/40 heteromeric gap junctions) may simultaneously be targeted by these kinases and PKA and thus exhibit a greater sensitivity to H/Rs than Cx40 homomeric gap junctions alone (37). Clearly, further work must be done to elucidate the mechanism of H/Rs-induced reduction in vascular cell coupling.

The present results imply that H/Rs or I/R quickly reduces intercellular communication along the vascular wall. On the basis of our mathematical model of arteriolar hemodynamic resistance (38) and on our arteriolar conduction data (Fig. 8, vehicle group), a 50% local constriction (i.e., approximate constriction caused by local KCl puff) is predicted to increase hemodynamic resistance by a factor of 5.1 during control time (i.e., –60 min), and only by a factor of 2.1 at 15 min of reperfusion. Therefore, reduced conduction after I/R could significantly compromise arteriolar ability to alter hemodynamic resistance and thus affect control of blood flow in the tissue.

In conclusion, we showed that hypoxia (0.1% O2, 1 h) plus abrupt reoxygenation (5–90 min) results in increased intercellular resistance (i.e., decreased electrical coupling) between mouse microvascular endothelial cells grown in monolayers. Significantly, abrupt reoxygenation does not reduce coupling between cells from Cx40−/− mice. We propose a model (Fig. 9) where abrupt reoxygenation elevates ROS production which, in turn, inhibits PKA activity and prevents PKA-dependent signaling that targets Cx40. This model is consistent with our discovery that ischemia (1 h) plus abrupt reperfusion (15–30 min) reduce arteriolar conducted response in the mouse cremaster muscle in PKA-dependent manner.

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Figure 1. Determination of coupling between mouse microvascular endothelial cells (MMEC). A) Electrical coupling. Example of dependence of membrane potential deflections on interelectrode distance (i.e., distance between current-injecting and recording electrodes, details in Methods) in MMEC monolayers in vitro (wild-type mouse hindlimb muscle origin). Solid symbols represent recordings for control monolayers, while open symbols are for monolayers exposed to hypoxia (H, 0.1% O2 for 1 h) followed by 10 min of abrupt reoxygenation, Ra (i.e., hypoxic cell culture medium replaced with normoxic medium at 19% O2). Lines represent best fit through the points. On the basis of a Bessel function model, the slope of each line represents the intercellular electrical resistance (i.e., inversely related to intercellular electrical coupling) of the monolayer. H/Ra increased interendothelial electrical resistance. B) Dye coupling. Example of fluorescence recovery in a photobleaching experiment in the monolayer. Cells were exposed to normoxia (solid symbols) or hypoxia (open symbols) for 1 h, and then exposed to normoxic culture medium together with 10 µg/ml 5(6)-carboxyfluorescein diacetate dye for 20 min. After this, the cell-averaged baseline fluorescence intensity in 4–5 cells was recorded for 20 s, and then these cells were photobleached with a laser beam to ~60% of the baseline intensity (i.e., to I_{bleach,control} and I_{bleach,H/Ra} values). The recovery of fluorescence in these cells was recorded for the next 5 min, to the final intensity values I_{5min,control} and I_{5min,H/Ra}. H/Ra reduced the fluorescence recovery when compared with control cells, indicating a reduced intercellular dye coupling.
Figure 2. Effect of hypoxia and abrupt reoxygenation (H/Ra) on expression of connexin 37 (Cx37), Cx40, and Cx43 in wild-type (WT) mouse microvascular endothelial cell monolayers. There was no change in expression of any of the connexins after hypoxia (0.1% O2, 1 h) followed by reoxygenation (30 min). β-actin blots served as a loading control. Blots shown are representative of at least 3 experiments.
Figure 3. Effect of H (1 h)/Rₐ (10–210 min) on intercellular resistance (A) and fluorescence recovery after photobleaching (B) in WT mouse monolayer. A) Resistance was increased during the first 90 min of abrupt reoxygenation, but it returned toward normoxic control level after 2 h of reoxygenation. *Significant difference from control. C. \( P < 0.05, n = 4 \) monolayers per group. B) Referring to Fig. 1B, the normalized fluorescence intensities immediately after photobleaching, \( I_{\text{bleach,control}} \) and \( I_{\text{bleach,H/Ra}} \) were 50 ± 5 and 52 ± 5 units, respectively. For each of the control and H/Rₐ groups, we computed the fluorescence recovery as \( (I_{\text{min}} - I_{\text{bleach}})/(100 - I_{\text{bleach}}) \times 100\% \). H/Rₐ significantly reduced recovery, indicating a reduced intercellular dye coupling. *Significant difference from control. \( P < 0.05, n = 5–6 \) monolayers per group.
Figure 4. Effect of hypoxia followed by abrupt or slow reoxygenation, deletion of Cx40, and activation/inhibition of PKA activity on intercellular resistance in MMEC monolayers. A) Bars 2 and 3 show the effect of H/Ra (5–60 min)(i.e., cells abruptly reoxygenated with fresh normoxic culture medium), and H/Rac (5–60 min)(i.e., cells abruptly reoxygenated with conditioned medium) on intercellular resistance. Details of fresh and conditioned medium are described in Methods. Abrupt reoxygenation with either medium increased resistance. Bar 4 shows that hypoxia followed by slow reoxygenation (H/Rs)(5–60 min) caused no change in resistance. Slow reoxygenation consisted of introduction of room air oxygen to cell monolayer via diffusion through ~2-mm-thick unstirred hypoxic cell culture medium. Bars 5 and 6 demonstrate the lack of effect of H/Ra (5–60 min) on intercellular resistance in Cx40⁻/⁻ monolayers. *Significant difference from the appropriate control group. \( P < 0.05, n = 28, 28, 4, 4, 8, \) and 8 for bars 1–6, respectively. B) WT monolayers pretreated with cell-permeable cAMP analog, 8-bromo cAMP (1 mM, 5 min), and subsequently exposed to H/Ra (5–60 min) yielded no increase in resistance (bars 7 and 8). Control WT monolayer pretreated with PKA inhibitor 6–22 amide (10 nM, 1 h) yielded an increase in resistance similar to that caused by H/Ra alone (bar 9 vs. 1). Agents remained in the culture medium during H/Ra. #Significant difference from control in bar 1. \( P < 0.05, n = 4 \) for each of bars 7–10.
Figure 5. Intracellular protein kinase A (PKA) activity in WT monolayers. H/R_a (30 min) decreased intercellular PKA activity (bars 1 and 2). One hour pretreatment with PKA inhibitor 6-22 amide (10 nM) inhibited activity in control (normoxic) cells, while it did not further reduce activity after H/R_a (bars 3 and 4). Five-minute pretreatment with PKA activator 8-bromo cAMP (1 mM) increased activity in both control and H/R_a-exposed cells (bars 5 and 6). (Note: These increases also reflected the inherent sensitivity of the PKA assay to 8-bromo cAMP). *Significant differences from control cells (bar 1), as determined by Dunnett’s post hoc test. \( P < 0.05 \), \( n = 5–6 \) monolayers per group in bars 1–6.
Figure 6. Effect of hypoxia followed by abrupt or slow reoxygenation, deletion of Cx40, and ascorbate on PKA activity and reactive oxygen species (ROS) formation. A) In both wild-type and Cx40<sup>−/−</sup> cells, hypoxia followed by an abrupt reoxygenation significantly decreased PKA activity (bar 2 vs. 1, and bar 5 vs. 4). In contrast, wild-type cells exposed to hypoxia followed by slow reoxygenation displayed no change in PKA activity (bar 3 vs. 1). Preloading of wild-type cells with the antioxidant ascorbate (200 µM for 4 h) also showed no change in PKA activity after H/R<sub>a</sub> (bar 7 vs. bars 6 or 1). *Significant difference from the appropriate control group. P < 0.05, n = 4–6 monolayers per group in bars 1–7. B) In both wild-type and Cx40<sup>−/−</sup> cells, hypoxia followed by abrupt reoxygenation resulted in increased intracellular ROS production. In wild-type cells, hypoxia followed by slow reoxygenation resulted in no increase in ROS, as compared with control (bar 3 vs. 1). (ROS could not be measured in cells preloaded with ascorbate, as ascorbate directly reacts with the ROS detection molecule NBT). *Significant difference from the appropriate control group. P < 0.05, n = 5 monolayers per group in bars 1–5.
Figure 7. Antioxidant ascorbate protects against H/Ra-induced increase in resistance. WT mouse monolayers were pretreated with ascorbate (200 µM for 4 h) or vehicle (PBS) and subsequently exposed to H/Ra (5–40 min). *Significant difference from control cells. $P < 0.05$, $n = 4$ monolayers per group.
Figure 8. Effect of ischemia and reperfusion (I/R) on KCl-induced conducted vasoconstriction in arterioles of WT mouse cremaster muscle in vivo. At –120 min baseline time point, the pre-KCl diameter was 63 ± 3 µm; local KCl puff of 142 ± 34 ms pulse duration reduced local diameter by 29.2 ± 1.4 µm to initiate the conducted response. The left bar (–120 min) represents the baseline-conducted response expressed in terms of the communication ratio, CR500, in 18 arterioles of 18 WT mice (details in Methods). The mice were randomly divided into 3 equal groups, and arterioles in each group were treated with vehicle (PSS), 8-bromo cAMP (10 mM, PKA activator), or 6-22 amide (100 nM, PKA inhibitor) between –120 and –60 min. Complete ischemia between –60 and 0 min was followed by abrupt reperfusion (i.e., clamp release) for 60 min. Regarding the local effect, there were no differences in the KCl pulse duration, pre-KCl diameter, or degree of local diameter reduction, between the baseline level at –120 min and any of the treatment groups or the time points shown, indicating that neither I/R nor the treatments altered arteriolar responsiveness to KCl. I/R reversibly reduced the conducted response. 8-bromo cAMP prevented this reduction, while 6-22 amide mimicked the effect of I/R in the preischemic arteriole (–60 min). *Significant difference from the baseline group at –120 min; P<0.05.
Figure 9. Proposed model of reduced interendothelial cell electrical coupling following hypoxia and abrupt reoxygenation. Baseline protein kinase A (PKA) activity keeps connexin (Cx) 40-containing gap junctions open to maintain coupling. Quickly after the onset of reoxygenation (i.e., within 5 min), elevated production of ROS inhibits PKA activity and reduces Cx40-dependent coupling. Deletion of Cx40, ROS inhibition or PKA activation after H/Ra prevents this reduction. PKA inhibition in normoxic cells mimics this reduction in coupling.