Compartmentalized Connexin 43 S-Nitrosylation/ Denitrosylation Regulates Heterocellular Communication in the Vessel Wall

Adam C. Straub, Marie Billaud, Scott R. Johnstone, Angela K. Best, Sean Yemen, Scott T. Dwyer, Robin Loof-Wilson, Jeffery J. Lysiak, Ben Gaston, Lisa Palmer, Brant E. Isakson

Objective—To determine whether S-nitrosylation of connexins (Cxs) modulates gap junction communication between endothelium and smooth muscle.

Methods and Results—Heterocellular communication is critical for endothelial control of smooth muscle constriction; however, the exact mechanism governing this action remains unknown. Cxs and NO have been implicated in regulating heterocellular communication in the vessel wall. The myoendothelial junction serves as a conduit to facilitate gap junction communication between endothelial cells and vascular smooth muscle cells within the resistance vasculature. By using isolated vessels and a vascular cell coculture, we found that Cx43 is constitutively S-nitrosylated on cysteine 271 because of active endothelial NO synthase compartmentalized at the myoendothelial junction. Conversely, we found that stimulation of smooth muscle cells with the constrictor phenylephrine caused Cx43 to become denitrosylated because of compartmentalized S-nitrosothiol reductase, which attenuated channel permeability. We measured S-nitrosothiol breakdown and NOX concentrations at the myoendothelial junction and found S-nitrosothiol reductase activity to precede NO release.

Conclusion—This study provides evidence for compartmentalized S-nitrosylation/denitrosylation in the regulation of smooth muscle cell to endothelial cell communication. (Arterioscler Thromb Vasc Biol. 2011;31:399-407.)

Key Words: NO ■ GSNO-R ■ connexin ■ myoendothelial junction ■ nitrosylation

Within the vessel wall of resistance arteries, coordinated vascular smooth muscle cell (SMC) and endothelial cell (EC) function is integrated by complex intercellular signaling to regulate the constriction and dilation of the artery. The anatomic structures that facilitate direct SMC and EC communication within the resistance artery are myoendothelial junctions (MEJs), which are cellular extensions from ECs or SMCs that project through the internal elastic lamina1–3 and link the plasma membranes of the 2 different cell types together. The gap junctions (GJs) at the MEJ provide a conduit for second messenger and electric signaling between the 2 cell types.2,4,5 For example, phenylephrine (PE) stimulation of SMCs induces inositol 1,4,5-triphosphate (IP3) generation and an increase in [Ca2+]i, concentrations, constricting the artery. It is thought that the IP3 progresses to the adjacent EC through GJs at the MEJ, initiating an increase in [Ca2+]i, and the release of NO to modulate the magnitude of vasoconstriction, thereby regulating the tone of the artery.6–8 Elucidation of the mechanisms regulating this process could provide novel insight into blood pressure regulation; however, the process remains uncharacterized.

GJs are intracellular signaling channels formed by 2 hexameric hemichannels, with each adjacent cell contributing 1 hemichannel. Connexin (Cx) proteins compose the channels, of which 4 different Cxs have been identified in the vasculature, with multiple studies demonstrating a potentially important role for Cx43 at the MEJ.9 Recent studies have demonstrated that GJ communication and trafficking of Cx43 are modulated by caveolae10–12 and caveolin-1,13 supporting the observation that caveolin-1 could regulate Cx43 trafficking to the MEJ.14 In addition to regulating Cx43, caveolin-1 also regulates, mobilizes, and organizes several proteins, including endothelial NO synthase (eNOS).14–17 Although eNOS has not been shown at the MEJ, it is possible that it resides in this location because of the caveola-rich environment.

NO participates in a plethora of physiological functions within the vessel wall, including vasodilation18 and posttrans-
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and GSNOR indirectly regulates the levels of S-nitrosylated proteins by reducing GSNO, thereby providing equilibrium between S-nitrosylated proteins and GSNO. Thus, mice deficient in GSNOR and in vitro studies have confirmed that GSNOR mediates multiple cardiovascular functions.

In this study, we provide evidence of an eNOS/GSNOR axis that regulates compartmentalized S-nitrosylation/denitrosylation of Cx43 permeability at the MEJ, thereby altering the constriction and dilatory response of a resistance artery. We propose that the specific cellular localization of proteins capable of dynamic S-nitrosylation/denitrosylation may be a template for heterocellular communication in general.

Methods

The supplemental materials (available online at http://atvb.ahajournals.org) provide expanded descriptions.

Mice

Wild-type mice, strain C57Bl/6 (Taconic), GSNOR−/−, and GSNOR+/− (originally described by Liu et al24), were all males aged approximately 8 to 10 weeks and were used according to the University of Virginia Animal Care and Use Committee (Charlottesville) guidelines.

Vessel Cannulation

Mice were euthanized with an intraperitoneal injection of pentobarbital (60 to 90 mg/kg). First-order thoracodorsal (TD) arteries, a pair of resistance arteries (diameter, approximately 245 μm) with extensive MEJs and endothelium-dependent hyperpolarization that supplies blood to spinotrapezoid muscle (supplemental Figure I), were isolated for cannulation.

Immunolabeling on Transmission Electron Microscopy Sections

TD arteries and cremaster, coronary, and mesentery vessels were isolated and processed for immunogold labeling and quantified as previously described.

Cell Culture, Isolation of In Vitro MEJ Fractions, and Biotin Switch Assay

The vascular cell coculture (VCCC) was constructed as previously described.4

In vitro MEJ fractions were isolated from the VCCC as previously described.31

Isolated EC, MEJ, and SMC fractions were subjected to the biotin switch assay as previously described.

Immunoblots, Immunostaining, and Antibodies

Proteins were resolved using 4% to 12% bis-Tris gels, transferred to nitrocellulose, and visualized and analyzed using an imager (Li-Cor Odyssey Imager), as previously described.24

Immunostaining on frozen sections of VCCC and HeLa cells was performed as previously described.55 All images were captured using a confocal microscope (Fluoview 1000).

Data on antibody source, application, concentration, and company of purchase are found in supplemental Table 1.

Statistics

Statistics were performed using computer software (Origin Pro 6.0). All experiments were analyzed using 1- or 2-way ANOVA, followed by the Bonferroni posttest for differences between treatments where indicated. P<0.05 was considered significant.

Results

NO and GJs Regulate Heterocellular Communication

As demonstrated in Figure 1A, the stimulation of a control TD artery with PE induces an initial constriction, followed by a redilation to bring the vessel back to near resting tone. We believe that this response is because of heterocellular communication between the SMCs and the ECs, specifically the generation of a second messenger by PE in SMCs (eg, IP3 or constriction), which traverses GJs at the MEJ to activate eNOS in the ECs (redilation). For this reason, we initially tested a GJ inhibitor (carbenoxylone, Figure 1A) and an NOS inhibitor (L-Nitro-Arginine Methyl Ester [L-NAME], Figure 1B) and found them both capable of significantly enhancing the constriction and inhibiting the redilation response. Because these data indicated that both Cxs and eNOS might be important regulators for intercellular communication between SMCs and ECs, we used transmission electron microscopy (TEM) and quantified the amount of Cx43 and eNOS present in the TD artery (Figure 1C and 1D). Cx43 was the only Cx enriched at MEJs (supplemental Figure IIA), indicating that it is likely a major contributor of GJ heterocellular communication from SMCs to ECs. This observation was also seen at the in vitro MEJ.31 The presence of eNOS was also localized to the MEJ (Figure 1D), a trend we found throughout vascular beds (supplemental Figure IIB). Normal rabbit serum confirmed the specificity of our gold bead staining (supplemental Figure IIC).

By using the VCCC, we also identified localized eNOS to the in vitro MEJ (Figure 1E and 1F). In contrast, neuronal NO synthase expression in TD MEJs (supplemental Figure IID through IIF), inducible NO synthase (supplemental Figure IIG through III), or another vasodilatory enzyme (cystathionine, supplemental. Figure III through IIII) was not detected. Last, we observed caveolin-1, a protein capable of trafficking both eNOS and Cx43,10–17 as being localized at the MEJ in TD arteries and in the VCCC (supplemental Figure IIIA through IIII).

Although eNOS was identified at the MEJ in vivo and in vitro, this was not indicative of its activity. Therefore, we applied L-NAME to vessels, measured resting tone in the absence of an agonist, and found a significant constriction (Figure 2A). These data coincided with the localization of phosphorylated S1177 eNOS at the MEJ in vivo and in vitro (Figure 2B and 2C) in the absence of any agonist. Phosphorylated S633 eNOS (active) was found at the MEJ in vivo and in vitro (Figure 2B and 2C) in the absence of any agonist. Phosphorylated S633 eNOS (active) was also observed at MEJs in vitro; however, phosphorylated T495 eNOS (inactive) was not observed (Figure 2D and 2E).

S-Nitrosylation of Cx43 on C271 Regulates GJ Communication

Because our ex vivo and in vitro evidence demonstrated basally active eNOS and the presence of Cx43 at the MEJ, the capacity
of Cx43 to be S-nitrosylated was tested. Initial studies confirmed Cx43 to be constitutively S-nitrosylated in isolated TD arteries and at the in vitro MEJ (Figure 3A), whereas Cx40, a Cx found at the MEJ in some instances, was not S-nitrosylated (supplemental Figure IVA). To identify which cysteines may be responsible for S-nitrosylation, we used HeLa cells (which do not express Cxs or eNOS) and transfected in either Cx43 or Cx43 with all of the cysteines in the C-terminal mutated to alanines (Cx43C260/271/298A). After treatment of transfected HeLa cells with GSNO, S-nitrosylated Cx43 was only detected in the nonmutated sample (Figure 3B). This was repeated on purified Cx43 C-terminal36 and Cx43C260/271/298A C-terminal peptides, which produced identical results to Cx43 proteins expressed in HeLa cells (Figure 3C). Thus, to identify whether S-nitrosylation was site specific, we generated Cx43 containing only 1 C-terminal cysteine. After GSNO treatment, only Cx43C260/298A was S-nitrosylated, indicating that C271 in the Cx43 C-terminal was the target for S-nitrosylation of Cx43 (Figure 3D). Functional changes as a result of S-nitrosylation were then tested in HeLa cells transfected with cysteine mutants by measuring the extent of Ca2+ wave propagation after uncaging of P(4(5))-1-(2-nitrophenyl)ethyl (NPE)-IP3. Calcium propagation rates were increased in response to GSNO for both Cx43 and Cx43C260/298A cells but unchanged in Cx43C260/271A-, Cx43C271/298A-, and Cx43C260/271/298A-expressing cells (Figure 3E). HeLa cells transfected without Cx43 did not propagate Ca2+ waves after pseudouncaging (supplemental Figure IVB). The differences in calcium wave propagation did not result from trafficking defects of the Cx43 mutations because all were effectively localized to the plasma membrane (supplemental Figure IVC through IVH). These data indicate that Cx43 and its GJ function are capable of being regulated by S-nitrosylation at C271.

Compartmentalized Denitrosylation of Cx43 at the MEJ

Because our previous results had demonstrated that Cx43 was extensively expressed at the MEJ and had the capacity to be S-nitrosylated, we tested whether PE stimulation could alter Cx43 S-nitrosylation. By using isolated TD arteries, we found a reduction in S-nitrosylation of Cx43 3 minutes after PE stimulation of mouse TD arteries with 50 μmol/L PE. Application of carbeneoxolone (50 μmol/L, A) and L-NAME (100 μmol/L, B) significantly enhanced PE-induced vasoconstriction in the TD arteries. C and D, Immuno-TEM analysis of Cx43 (C) and eNOS (D) localization labeled with 10-nm gold beads (arrows) at MEJs from the TD arteries quantified the number of beads per micrometer squared. E, Isolated EC, MEJ, and SMC protein fractions from the VCCC blotted for eNOS and normalized to GAPDH. F, Immunocytochemistry of transverse sections from a VCCC were labeled for eNOS (green). The white box illustrates an enlarged MEJ with a line scan measuring fluorescence down the pore. Data are represented as the mean±SE. (C, n=8; D, n=6; E, n=4). Significant differences (P<0.05) were analyzed using a 2-way ANOVA (A and B) or a 1-way ANOVA (C-E). In A and B, n is the number of vessels and the value in parentheses is the number of mice. E indicates endothelial cell; IEL, internal elastic lamina; S, smooth muscle cell; *, lumen. The scale bar in C and D is 0.5 μm; and F, 10 μm. In C through E, the open bars indicate in vitro measurements; and bars with horizontal lines, in vivo measurements.
stimulation, which returned to baseline after 10 minutes (Figure 4A). This result indicated that, in vivo, Cx43 at the MEJ is likely S-nitrosylated. Concurrent with this result, denitrosylation of Cx43 was only observed at the MEJ, and not the EC or SMC monolayer (Figure 4B), indicating a highly localized denitrosylation response (an effect identical to that seen on silver-stained gels of total S-nitrosylated proteins after PE stimulation) (supplemental Figure VA through VC). Neither application of 18GA (supplemental Figure VD) nor the UV used for uncaging (supplemental Figure VE) altered Cx43 S-nitrosylation. To test whether denitrosylation of Cx43 correlated with changes in channel permeability, we stimulated SMCs on the VCCC with PE and then temporally uncaged NPE-IP3 (Figure 4C). Under control conditions, uncaging of NPE-IP3 in the SMCs elicited a robust increase in EC [Ca$^{2+}$]i, which was significantly inhibited by the GJ blocker (18GA, Figure 4D). At 1 minute after PE stimulation, there was a significant reduction in EC [Ca$^{2+}$]i (Figure 4E).
which returned to control levels at 20 minutes after PE stimulation. This suggested that the permeability of the GJ channel immediately after PE stimulation was decreased, which was likely because of a loss of Cx43 S-nitrosylation.

GSNOR Denitrosylates Cx43 at the MEJ

Because of the rapid denitrosylation of Cx43 at the MEJ on PE stimulation, we hypothesized that an enzyme capable of denitrosylating proteins may also be localized to the MEJ. Probing for GSNOR, we found the enzyme to be enriched in MEJ fractions both in vitro and in vivo (Figure 5A through 5C). In contrast, other enzymes known to denitrosylate proteins, thioredoxin-1,37 and carboxyl reductase,38 were not present at the MEJ (supplemental Figure VIA through VIC).

Next, we tested the activity of GSNOR after PE stimulation specifically in MEJ fractions and found increased activity at 1 minute, which returned to baseline after 20 minutes (Figure 5D). From the same MEJ lysates, we also measured total NOx and found a significant increase at 20 minutes compared with control and 1 minute (Figure 5E), suggesting that GSNOR activity precedes NO release. To test the effect of GSNOR activity on Cx43 denitrosylation and GJ permeability at the MEJ, we used the GSNOR inhibitor that was identified in a high-throughput screen for GSNOR inhibitors and thereby arbitrarily named C3.28 We found a complete lack of Cx43 denitrosylation after PE stimulation (Figure 5F), a result that was similar to the result obtained using GSNOR small-interfering RNA (supplemental Figure VIIA and VIIB).

Consistent with lack of denitrosylation after inhibiting GSNOR, application of C3 did not alter GJ permeability of IP3 from SMCs to ECs when compared with control (Figure 5G). TD arteries treated with C3 had an attenuated constriction after application of PE (Figure 5H). The C3 did not alter baseline artery diameter during equilibration (supplemental Figure VIIC). The GSNOR−/− mice were also less responsive to PE (Figure 5I), which was dependent on NOS activity and not S-nitrosothiols (supplemental Figure VIIID). Last, the GSNOR−/− mice were not different from controls (supplemental Figure VIIIE), a result that is due to compensatory increases in carboxyl reductase in the TD arteries (supplemental Figure VIIIF).

Discussion

Highly coordinated EC and SMC cross talk regulates vessel diameter and, by extension, the blood flow rate and blood pressure. GJs positioned at the MEJ between ECs and SMCs in resistance arteries allow for signals (eg, IP3) originating from 1 cell type (eg, SMCs) to rapidly diffuse to adjacent cells (eg, ECs). Although GJs have been identified at the MEJ, the specific mechanisms that regulate GJ communication at the MEJ remain largely unknown. We define a pivotal posttranslational mechanism that ECs and SMCs use to regulate heterocellular communication before, during, and after SMC constriction. Our mechanism consists of compartmentalized S-nitrosylation/denitrosylation of Cx43 at the MEJ to regulate the magnitude of vasoconstriction (Figure 6).
Several compelling observations support this discovery: (1) eNOS is enriched and active at the MEJ, (2) Cx43 S-nitrosylation on cysteine 271 regulates more permeable GJ channels, and (3) compartmentalized GSNOR denitrosylates Cx43, promoting less permeable GJs at the MEJ to modulate the movement of IP₃ (and potentially other factors). The cellular, pharmacological, and genetic results presented herein imply that oxidation-reduction–based protein modifications on site-specific cysteine residues are regulated in specific regions of cells to coordinate hetero-cellular communication.

SMC relaxation after PE-induced constriction is thought to be due to IP₃ movement from SMCs, through GJs at the MEJ, to ECs.⁷ There is evidence to indicate that the IP₃ receptors localized to the MEJ³⁵ and induces an elevation of EC [Ca²⁺], after UV uncaging is plotted at 0, 1, and 20 minutes after PE stimulation at the VCCCs pretreated with C3. H and I, Vasoconstriction response measuring percentage change of initial diameter to PE in TD arteries pretreated with C3 in wild-type mice (H) and GSNOR−/− mice (I). Data are represented as the mean±SE. (A, n = 5; C, n = 5; E, n = 2; and F, n = 3). In H and I, n is the number of vessels and the value in parentheses is the number of mice. Significant differences (*P<0.05) were analyzed using a 1-way (E–G) or a 2-way (H–I) ANOVA. The scale bar in B is 10 μm; and in C, 0.5 μm. E indicates endothelial cell; IEL, internal elastic lamina; S, smooth muscle cell; *, lumen. Open bars indicate in vitro measurements (A, E, and F); and bars with horizontal lines, in vivo measurements (C).

Figure 5. GSNOR regulates heterocellular communication. A, Quantitative Western blot analysis of GSNOR expression in isolated EC, MEJ, and SMC protein fractions from the VCCC normalized to GAPDH. Immunocytochemistry of transverse sections of a VCCC labeled for GSNOR (red). B, The white box illustrates an enlarged MEJ with a line scan measuring fluorescence down the pore. C, Immunocytochemistry of ECs expression labeled with 10-nm gold beads (arrows) at MEJs from the TD arteries and quantified as the number of beads per micrometer squared. D, Measurement of GSNOR activity by breakdown of GSNO in MEJ fractions at 1 and 20 minutes after PE stimulation. E, Identification of total NO, in MEJ fractions at 1 and 20 minutes after PE stimulation. F, Immunoblot of S-nitrosylated Cx43 from in vitro MEJ fractions pretreated with C3 inhibitor and then stimulated with PE for 0, 1, 5, 10, and 20 minutes. G, Measurement of maximum values of EC [Ca²⁺], after UV uncaging is plotted at 0, 1, and 20 minutes after PE stimulation from the VCCCs pretreated with C3. H and I, Vasoconstriction response measuring percentage change of initial diameter to PE in TD arteries pretreated with C3 in wild-type mice (H) and GSNOR−/− mice (I). Data are represented as the mean±SE. (A, n = 5; C, n = 5; E, n = 2; and F, n = 3). In H and I, n is the number of vessels and the value in parentheses is the number of mice. Significant differences (*P<0.05) were analyzed using a 1-way (E–G) or a 2-way (H–I) ANOVA. The scale bar in B is 10 μm; and in C, 0.5 μm. E indicates endothelial cell; IEL, internal elastic lamina; S, smooth muscle cell; *, lumen. Open bars indicate in vitro measurements (A, E, and F); and bars with horizontal lines, in vivo measurements (C).
at the MEJ (ie, regulation of GJ-mediated intracellular communication by NO). There are sporadic reports that NO could alter the function of GJ channels. For example, NO reduced Cx37 permeability and electric coupling in microvascular cells,\(^{41,42}\) whereas other reports\(^{43,44}\) suggest that NO enhances Cx43 electric current. However, this study used NO donors and did not explore how NOS-derived NO may posttranslationally modify the channel. It is becoming increasingly clear that S-nitrosylation is a critical posttranslational modification that regulates protein function.\(^{20,21}\) Our study demonstrates that NO derived from eNOS at the MEJ constitutively S-nitrosylates Cx43 in unstimulated conditions in TD arteries and in the VCCC, thereby maintaining a more permeable GJ channel. These data correlate with recent reports\(^{43,44}\) that NO acts on Cx43 hemichannels (not intact GJs) via S-nitrosylation to induce a more permeable state. Although the exact cysteines were not identified, these reports did show that the cysteines were more likely intracellular than extracellular. Therefore, we created several point mutations on the C-terminal of Cx43 and identified C271 as the critical site that significantly enhanced calcium wave propagation after IP3 uncaging. The sum of these data indicates that Cx43 S-nitrosylation on C271 enhances permeability of the GJ channel; this can occur in a discreet cellular compartment.

Although the aggregate of our work indicated that S-nitrosylation maintained a more permeable GJ channel, it was reasonable to propose that denitrosylation of Cx43 modulated a less permeable GJ channel. Remarkably, we observed that denitrosylation after PE stimulation was confined to the MEJ rather than the EC or SMC monolayer. Of the multiple enzymes that have regulated denitrosylation, including GSNOR/GSNO,\(^{24,45}\) thioredoxin-1,\(^{37}\) and carboxyl reductase,\(^{38}\) we found that GSNOR was the dominant enzyme localized at the MEJ. This was evident because GSNOR activity specifically at the MEJ increased immediately after PE stimulation, which returned to baseline after 20 minutes. Conversely, we found that NOx was increased only at the 20-minute point, supporting the idea that GSNOR activity precedes eNOS activity. It is unknown how GSNOR activity is regulated, although one likely possibility is through a Ca\(^{2+}\)/H\(_{1001}\)-dependent signaling pathway based on the rapid IP3-induced increase in EC [Ca\(^{2+}\)]\(_{100}\) and the immediacy of the effect. The pharmacological and genetic approaches in this study also support that GSNOR regulates denitrosylation at the MEJ. In cannulated vessels, C3 and GSNOR\(^{-/-}\) mice both had severely attenuated constriction. It is not clear how this occurs, but based on the data from the VCCC, we believe this is due to the enhanced GJ permeability, allowing for greater IP3 transfer from SMCs to ECs enhancing eNOS-derived NO. Previous studies\(^{24,28}\) have demonstrated that inhibition of GSNOR increases S-nitrosothiols, thereby promoting SMC relaxation. However, our model system does not support this because L-NAME increased the magnitude of the constriction in cannulated vessels. Rather, these data confirm that NOS activity is a critical modulator of SMC constriction. These observations underpin the central role that compartmentalized GSNOR plays in regulating heterocellular communication in the artery wall.

In summary, results from this study emphasize the critical role that S-nitrosylation/denitrosylation contributes to heterocellular communication. Specifically, the evidence provided herein supports multiple roles for eNOS at the MEJ, including the following: (1) basal release of NO for regulating vasoconstriction, and (2) local S-nitrosylation of...
Cx43 to regulate more permeable channels, and (3) local production of NO for immediate feedback on SMCs. The presence of GSNO provides a check on this system by inducing Cx43 denitrosylation on constriction and inducing a less permeable GJ. The results provide an investigational framework for future endeavors focusing on the eNOS/GSNO axis as a potential therapeutic target for treating vascular pathological features, such as hypertension.

Acknowledgments
We thank Mark Yeager, PhD, MD, Brian Duling, PhD, Michael Koval, PhD, and Aaron Barchowsky, PhD, for critical reading and discussion of the manuscript; John Hunt, MD, for use of the NO analyzer; the University of Virginia Histology Core for sectioning of VCCCs; and Jan Redick, BS, and Stacey Guillot, PhD, at the Advanced Microscopy Core.

Sources of Funding
This study was supported by a postdoctoral fellowship from the National Research Service Award (NRS) (Dr Straub); postdoctoral fellowships from the American Heart Association (Drs Billaud and Johnstone); grants HLO82647 (Dr Looft-Wilson), HL59337 and HL69170 (Dr Gaston), and HL088554 (Dr Isaakson) from the National Institutes of Health; Department of Defense (DOD) W81VWH-07-1 to 0134 (Dr Palmer); and American Heart Association Scientist Development Grant (SDG) (Dr Isaakson).

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2011;31:399-407; originally published online November 11, 2010;
doi: 10.1161/ATVBAHA.110.215939

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplemental Figure Legends

Supplemental Figure I. Dose response curve to acetylcholine in TD arteries in presence of L-NAME.

Mouse TD arteries were cannulated, pressurized and stimulated with PE (10^{-5}M) followed by increasing concentrations of acetylcholine in the presence or absence of L-NAME. Significant differences of p<0.05 were analyzed using a 2-way ANOVA and are indicated by *. N = number of vessels, ( ) are the number of mice.

Supplemental Figure II. Localization of Cxs, eNOS, nNOS, iNOS, and CTH at the MEJ.

(A) Connexin localization at the MEJ. Quantification of gold beads for each connexin were quantified using Metamorph. Data are represented as the mean ±SE (n=6). Scale bar equals 0.5 µm.

(B) eNOS localization at the MEJ. Isolated cremaster, coronary, mesentery vessels were immunolabeled for eNOS expression using 10 nm gold beads. Gold beads were quantified for eNOS expression in ECs, MEJs, and SMCs were measured by averaging the number of total eNOS beads per micron squared. Data are represented as the mean ±SE (n=6).

(C) Negative control for immuno-TEM labeling. A representative image of a TD artery incubated with normal rabbit serum followed by anti-rabbit 10 nm gold beads. Scale bar equals 0.5 µm.

(D-F) nNOS localization at the MEJ. Immuno gold labeling for nNOS expression at the MEJ and bead quantification in vivo (D-E). Quantitative western blot analysis of nNOS expression normalized to GAPDH in the VCCC (F). Data are represented as the mean ±SE (E n=6). Scale bar equals 0.5 µm.

(G-I) iNOS expression at the MEJ. Immuno gold labeling for iNOS localization at the MEJ and bead quantification in vivo (G-H). Quantitative western blot analysis of iNOS expression normalized to GAPDH in the VCCC (I). Data are represented as the mean ±SE (H n=6). Scale bar equals 0.5 µm.
(J-L) CTH localization at the MEJ. Immuno-gold labeling for CTH expression at the MEJ and bead quantification in vivo (J-K) and quantitative western blot analysis of CTH expression in the VCCC (L). Data are represented as the mean ±SE (K n=5; L n=3). Scale bar equals 0.5 μm.

Supplemental Figure III. Caveolae and caveolin-1 localization to the MEJ.

(A-E) Caveolae and cav-1 localization at the MEJ. A representative TEM image from a TD artery at low magnification highlighting multiple MEJs (A), with a magnified section (black box) indicating a MEJ. The magnified black box in (B) illustrates multiple caveolae-like vesicles identified with black arrows. Immuno-TEM analysis of cav-1 localization using 10 nm gold beads (arrow) at the MEJ in TD arteries and quantified as the number of beads per micrometer squared (C). Quantitative western blot analysis from EC, MEJ, and SMC fractions for cav-1 normalized to GAPDH expression in the VCCC (D). Immunocytochemistry of a transverse section from the VCCC labeled for cav-1 (green) (E). The white box illustrates enlarged MEJ with a line scan measuring fluorescence down the pore. Enlarged picture of pore illustrated at left edge of line scan. Data are represented as the mean ±SE (C n=7; D n=4). Scale bar equals 0.5 μm (A-C) and 10 μm (E). E: endothelial cell, S: smooth muscle cell, IEL: internal elastic lamina, #: lumen.

Supplemental Figure IV. S-nitrosylation of Cx40, Ca^{2+} propagation in untransfected HeLa cells, and trafficking of Cx43 cysteine mutants.

(A) Connexin 40 is not S-nitrosylated at MEJ. Unstimulated MEJ fraction were isolated and subjected to the biotin switch assay and blotted for Cx40.

(B) Ca^{2+} propagation in untransfected HeLa cells. Untransfected HeLa cells were loaded with NPE-IP$_3$, treated with GSNO for 1hr followed by UV flash to uncage IP$_3$ to determine Ca^{2+} wave propagation. Data are represented as the mean ±SE (n=6-8).
Supplemental Figure V. Total protein denitrosylation at the MEJ and unaltered Cx43 S-nitrosylation using the GJI and UV flash.

(A-C) Total denitrosylated proteins at the MEJ after PE stimulation. Silver stain of total S-nitrosylated proteins using the biotin switch assay from EC, MEJ, and SMC protein fractions treated with PE in the VCCC for 0, 1, 5, 10, and 20 minutes. Using Metamorph, a line scan down each well measured grayscale intensity and was plotted for each time point for each of the isolated fractions.

(D) Connexin 43 S-nitrosylation is unchanged with GJI. VCCCs were pretreated with 18GA for 1 hr and stimulated with PE for 0, 1, 5, 10, and 20 minutes. MEJ fractions were isolated and subjected to the biotin switch assay.

(E) UV flash does not alter Cx43 S-nitrosylation. Western blot analysis of VCCC fractions blotted for S-nitrosylated Cx43 after UV flash.

Supplemental Figure VI. Trx-1 and CBR1 expression at the MEJ in the VCCC.

(A-C) Trx-1 and CBR1 expression at the MEJ. Quantitative western blot analysis of Trx-1 and CBR1 expression from EC, MEJ, and SMC fractions normalized to GAPDH.

Supplemental Figure VII. GSNOR siRNA treated VCCCs, changes in baseline diameter after with pharmacological inhibitors, vasoreactivity changes in GSNOR−/− mice and up-regulation of CBR1 in TD arteries.
(A) Knockdown efficiency of GSNOR siRNA. Isolation of MEJ fractions from VCCC treated with or without GSNOR siRNA for 48hrs and subjected to western blot analysis for knockdown of GSNOR protein.

(B) GSNOR siRNA prevents Cx43 denitrosylation after PE stimulation. Vascular cell co-cultures were pretreated with GSNOR siRNA for 48hrs and stimulated with PE for 0, 1, or 20 min. MEJ fractions were isolated, subjected to the biotin switch assay, and western blotted for Cx43.

(C) Baseline diameter changes of TD artery in WT treated with pharmacological inhibitors. The percent change is baseline before and after 30 minute equilibration time with CBX and C3. Data are represented as the mean ±SE (n=5).

(D) Vascular reactivity changes in GSNOR^{+/−} TD arteries pretreated with L-NAME (100µM). Isolated TD arteries from GSNOR^{+/−} mice were cannulated, pretreated with L-NAME and stimulated with PE.

(E) Vascular reactivity changes in GSNOR^{−/−} mice. Isolated TD arteries from GSNOR^{+/−} mice were cannulated and stimulated with PE (10µM). Data are represented as the mean ±SE (n=8).

(F) Carboxyl reductase expression from isolated TD arteries from WT, GSNOR^{+/−}, GSNOR^{−/−}. Total protein was isolated from TD arteries and western blotted for CBR1.
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Supplemental Figure VII

A

GSNOR

GAPDH

GSNOR/GAPDH

(Integrated intensity)

control

GSNOR siRNA

B

SNO-Cx43

total Cx43

Minutes after PE stimulation

c
1
20

D

Controls

GSNOR (+/−) + L-NAME 100 μM

C

Percent change in baseline diameter after 30 minute equilibration

Control

CBX

C3

E

Controls n=8 (6)

GSNOR −/− n=9 (9)

% of initial inner diameter

minutes after 10 μM PE stimulation

F

CBR1

Tubulin

CBR1/tubulin (integrated intensity)

+/+

−/+ −/−
Supplemental Methods:

**Cell Culture:** Human umbilical vein endothelial and vascular smooth muscle cells were obtained from Cell Applications Inc. and cultured in M199 media supplemented with 10% fetal bovine serum (Gibco), 2mM L-glutamine (Gibco), penicillin (2mM)/streptomycin (50 U/mL) (Gibco). Endothelial cell medium was further supplemented with endothelial growth supplement (5µg/mL, BD Biosciences) and 5µg/mL of sodium heparin (Fisher Scientific). Vascular cell co-culture studies using the pharmacological inhibitors 18α glycyrrhetinic acid (18GA) (Sigma) and C3 was performed by pretreating EC and SMC with 50µM for 1 and 3 hours respectively, followed by treatment with PE (50µm). HeLa parental cells (obtained from UVA cell culture core) were maintained in complete DMEM (cDMEM) (Gibco) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 (U/mL) and L-glutamine (50 U/mL).

**Vessel Cannulation:** Mice were sacrificed with an intraperitoneal injection of pentobarbital (60-90 mg/kg) and isolated first order thoracodorsal (TD) arteries were cannulated at both ends with glass micropipettes, secured with 10–0 nylon monofilament suture and placed in a pressure myograph (Danish MyoTechnology). The chamber was superfused and perfused with Kreb’s-Hepes solution, gassed with air, and maintained at 37°C. Vessels were maintained in a no-flow state and held at a constant transmural pressure of 80mm Hg equivalent to the transmural pressure of these vessels in vivo. Vessels were pretreated with carbenoxolone (CBX) (50µM) (MP Biomedical), L-Nitro-Arginine-Methyl Ester (L-NAME) (100 µM) (Sigma) or C3 (50µM) (Chem Div) described in for 30 minutes and baseline diameters were measured after 30 min equilibration using an Olympus Fluoview 1000 confocal microscope. Contractile responses to 10 or 50 µM of PE were measured and recorded every one minute for a total time of 15 minutes and vessel diameter changes were quantified using MetaMorph as described in. Post PE stimulation, all vessels were tested for functional endothelium by stimulating with 10µM acetylcholine to ensure maximal dilation.
**siRNA:** Endothelial cells and SMCs were cultured together for 24hrs on Transwells and transfected with pre-designed siRNAs (5nM) for GSNOR (Ambion) using siPORT™ NeoFX™ transfection Agent (Ambion) according to manufactures directions. After 48hrs post transfection of siRNA, MEJ fractions were treated with PE and harvested for knockdown efficiency or subjected to the biotin switch assay. The following siRNA sequences were purchased from Ambion: siRNA ID# s1072-sense 5’AUUUGUCUAAAUCCUAAtt 3’, antisense 5’UUUAGGAUUUGACAAAAUt 3’, and siRNA ID#s1070 sense 5’AAAUCAAAGCCUUUGAtt 3’ and antisense 5’UCAAAGGCUUUGUUGAUUca 3’.

**Transfection:** Parental Hela cells were transfected at 70% confluency with Cx43 plasmids described below using a Nucleofector (Lonza) achieving an approximate 70% transfection efficiency. Transfected cells were maintained in cDMEM supplemented with G418 (800 µg/mL) for 48hrs.

**Biotin Switch Assay:** Briefly, samples were isolated, acetone precipitated, and blocked with 10mM N-ethylmaleimide (ACROS Organics) at 50°C for 30 min. Following an additional acetone precipitation, samples were incubated with biotin-HPDP-N-[6-(biotinamido)hexyl]-3’-(2’-pyridyldithio) propionamide (Peirce) along with 1mM sodium ascorbate with the addition of 10µM copper sulfate for 1hr at RT as described in 33. Samples were then precipitated and incubated with streptavidin coated agarose beads for 1 hr, washed 5 times, and S-nitrosylated proteins were eluted and subjected to immunoblotting.

**Silver Stain:** After protein separation, gels were fixed with 50% ethanol and 5% acetic acid overnight followed silver staining according to manufactures instructions (ThermoFisher).

**Calcium Signaling after IP3-uncaging:** Both the SMC and HeLa cells were loaded with NPE-IP3 (300 µM) using the pinocytotic method as previously described 4. When using the VCCC, uncaging of the NPE-IP3 in the SMC was achieved by two synchronized xenon light sources under the control of Slidebook software. The UV-flash was transmitted through a centered 25 µm pinhole at 1, 5, 10 or 20 min post-stimulation of SMC with PE. Simultaneous measurement of changes in EC [Ca2+]i on the VCCC was performed as described 31, utilizing a Hamamatsu 9100-13 back-thinned (512x 512) CCD camera. Transfected HeLa cells were initially loaded with P(4(5))-1-(2-nitrophenyl)ethyl-caged IP3.
Calcium wave propagation occurred radially outward immediately upon uncaging of NPE-IP$_3$ from the spatially defined UV-flash 25 µm spot (as above). The distance of the calcium wave propagation was measured 10 seconds after UV-flash for each of the Cx43 mutants transfected into HeLa cells.

**Generation of Cx43 C-terminal mutant peptides:** The Cx43 C-terminal encoding the sequence 236-382 of rCx43 inserted into pGEX-6P-2 plasmid was kindly provided by Dr. Paul Sorgen (University of Nebraska) and was synthesized as described. Cx43 C-terminal mutations were made via site directed mutagenesis with cysteine to alanine primers designed against the three C-terminal domain cysteines at C25, C36 and C63 in the Cx43 C-terminal sequence corresponding to C260, C271, C298 respectively in the full length Cx43 sequence using primers described above. For mutagenesis reactions the Cx43 C-terminal insert was removed from the pGEX-6P-2 by digest with Bam-H1 and Xho-1 followed by gel extraction of the insert, which was then ligated into pBluescript plasmid. Following mutagenesis, the insert was removed from pBluescript and re-ligated into pGEX-6P-2 plasmid as described above.

**Cx43 C-terminal peptide:** Proteins were purified as described with the following alterations to the protocol. Plasmids containing the Cx43 C-terminal sequence were transformed into BL21 competent bacterial cells. Bacterial clones grown in 2L of LB broth were induced at 0.5 OD with 1mM isopropyl-1-thio-B-D-galactopyranoside (IPTG) for 3 hours. Bacterial pellets were washed in PBS pH 7.4 and re-suspended (1g/5mL) in protein buffer (50mM Tris-HCL (pH7.4), 190mM NaCl, 1mM dithiothreitol (DTT), 0.1mM Pefabloc, 200U of DNAse, 1% Glycerol and 1:100 dilution of complete protease inhibitor cocktail (Sigma) then stored at -80°C. Cells were then thawed and disrupted by standard French Pressure 20K then lysates cleared by centrifugation (100,000G, 1 hr, 4°C). Supernatants were incubated with 4mL of glutathione-sepharose beads (Glutathione-sepharose 4 fast flow) for 3 hrs at room temperature. Protein bound beads were washed with 40 column volumes of protein buffer, followed by 20 column volumes of protein buffer without Pefabloc or protease inhibitors and Cx43-GST cleavage performed on the beads with PreScission (80U) at 4°C overnight with rocking. Eluted Cx43 C-terminal peptides were then further purified by incubation with fresh glutathione-sepharose beads for 3 hrs to remove residual
unbound GST, flow through was collected and protein samples were spin concentrated using Amicon centriplus 10 filters. Final Cx43 C-terminal proteins were found to be approximately 80% pure. Cx43 C-terminal were then treated with 100µM GSNO at 37ºC shaking for 1hr and subjected to the biotin switch assay.

**Site-directed Mutagenesis:** Mutants were generated using Quickchange (Stratagene) site-directed mutagenesis kit according to manufactures directions using Cx43 in a pcDNA 3.1 plasmid (gift from Mike Koval, Emory University). Primers for C260A are as follows: forward 5’ ctgagcccatcaaagagcggagctctcctcaagaagcgc 3’ reverse 5’ gcgtattttggagatccggcgtcttttgatggctcag 3’; C271A: 5’ gccctacttcaatggcgcctcctcaccaacggc3’, reverse 5’ gccttgttttggaggagggcgcattgaagagc 3’; and C298A forward 5’gtgacagaaactcctcgggccgaattacaacaagcaag 3’, reverse 5’ cttgcttttgatgcttggaggaatgtttctgc 3’. Mutants were sequenced at the UVA DNA sequencing core, confirmed and maxi prepped for transfection studies. The following mutants were generated Cx43 C260/271/298A, Cx43 C260/271A, Cx43 C260/298A, and Cx43 C271/298A.

**GSNOR activity assay:** GSNO-R activity was measured by using a modified Saville Assay. Briefly, 300µg of MEJ lysate was incubated with 300µM NADH, 2mM GSH and 28µM GSNO. Two aliquots of 75µl were placed into a 96 well plate at 1 min intervals for a total of 5 min. One aliquot was placed with 75µl of (+) reagent (58 mM Sulfanilamide + 7.36 mM HgCl2 in 1N HCl) while the second aliquot was placed with (-) reagent (58 mM Sulfanilamide in 1N HCl). Samples were incubated 5 min in the dark. At the end of this incubation, 75µl of (N) reagent (0.77M n-(1-napthyl) ethylene-diamine dihydrochloride) was added. Samples were incubated 5-10 min for color to develop. Absorbance was read at 540nm. Amount of GSNO remaining in the reaction was determined from a GSNO standard curve. Activity was obtained from the slope of the time course divided by the amount of protein in the reaction.

**Determination of NO metabolites:** Total nitrate and nitrite (NOx) were analyzed according to manufactures instructions (Nitric oxide Analyzer, Sievers). Briefly, 2.5 µg of MEJ lysate diluted in a final volume of 50 µL was injected into a purge vessel containing a solution of vanadium (III) chloride
(50 mmol/L) in hydrochloric acid (1 mol/L) at 95°C. A continuously purged stream of nitrogen gas connected to a Sievers 280i NO analyzer measured NO$_x$. 