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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 Looft-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 essential for endothelium 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-nitrosoglutathione reductase, which attenuated channel permeability. We measured S-nitrosoglutathione breakdown and NO concentrations at the myoendothelial junction and found S-nitrosoglutathione 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-
lational protein S-nitrosylation. S-nitrosylation has emerged as a key mechanism by which NO may influence the function of a wide array of cellular proteins by covalently modifying sulfhydryl groups on cysteine residues both in vivo and in vitro. Despite its high ability to diffuse, growing evidence supports the concept that NO is regulating the balance between protein S-nitrosylation and protein denitrosylation is essential. One enzyme involved in regulating the balance between protein S-nitrosylation/denitrosylation is S-nitrosoglutathione reductase (GSNOR). GSNOR indirectly regulates the levels of S-nitrosylated proteins by reducing GSNO, thereby providing equilibrium between S-nitrosylated proteins and GSNO. Indeed, 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. In vitro MEJ fractions were isolated from the VCCC as previously described. 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. Immunostaining on frozen sections of VCCC and HeLa cells was performed as previously described. All images were captured using a confocal microscope (Fluoview 1000).

Statistics

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 (carbenoxolone, Figure 1A) and an NO 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. 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 IIH), 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, as being localized at the MEJ in TD arteries and in the VCCC (supplemental Figure IIIA through IIIE).

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 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-terminal 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, 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...

Figure 2. eNOS is differentially phosphorylated at the MEJ. Mouse TD arteries were cannulated and pressurized. A, The basal vascular tone was attenuated in the presence of L-NAME (100 \(\mu\)mol/L). B, Phosphorylated eNOS S1177 localization using 10-nm gold beads (arrow) in TD arteries using immuno-TEM and quantified as the number of beads per micrometer squared. C through E, isolated EC, MEJ, and SMC fractions from the VCCC blotted for phosphorylated eNOS at sites S1177, S633, and T495 in EC, MEJ, and SMC fractions. Data are represented as the mean \(\pm\) SE. (A, n=5; B-E, n=4). Significant differences (*\(P<0.05\)) were analyzed using a 1-way ANOVA (A–D). E, endothelial cell; IEL, internal elastic lamina; S, smooth muscle cell; *, lumen. The scale bar is 0.5 \(\mu\)m (B). In A through E, open bars indicate in vitro measurements; and bars with horizontal lines, in vivo measurements.

Figure 3. Cx43 is S-nitrosylated on cysteine 271. A, Unstimulated TD arteries or MEJ fractions analyzed by the biotin switch assay for Cx43, in which ascorbate-dependent labeling demonstrates the presence of an S-nitrosylated cysteine residue(s). B, Nontransfected and transfected HeLa cells with Cx43 or Cx43C260/271A, 298A were treated with or without 100-\(\mu\)mol/L GSNO for 1 hour. C, Biotin switch assay of purified Cx43 C-terminal or Cx43C260/271/298A C-terminal peptides treated with or without 100-\(\mu\)mol/L GSNO for 1 hour. D, HeLa cells transfected with Cx43C260/271A, Cx43C260/298A, or Cx43C271/298A and treated with 100-\(\mu\)mol/L GSNO for 1 hour were lysed and subjected to the biotin switch assay. E, Uncaging of NPE-IP3 and analysis of calcium wave propagation and transfected HeLa cells with Cx43, Cx43C260/271/298A, Cx43C260/271A, Cx43C260/298A, and Cx43C271/298A. Data are represented as the mean \(\pm\) SE (n=6 to 8). Significant differences (*\(P<0.05\)) were analyzed using a 1-way ANOVA.
[Ca\textsuperscript{2+}], 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,\textsuperscript{37} and carboxyl reductase\textsuperscript{38} were not present at the MEJ (supplemental Figure VI A 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 NO\textsubscript{x} 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.\textsuperscript{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 VII A and VII B).

Consistent with lack of denitrosylation after inhibiting GSNOR, application of C3 did not alter GJ permeability of IP\textsubscript{3} 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 VI C). The GSNOR\textsuperscript{−/−} mice were also less responsive to PE (Figure 5I), which was dependent on NOS activity and not S-nitrosothiols (supplemental Figure VII D). Last, the GSNOR\textsuperscript{−/−} mice were not different from controls (supplemental Figure VII E), a result that is due to compensatory increases in carboxyl reductase in the TD arteries (supplemental Figure VII F).

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, IP\textsubscript{3}) 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 activates IP3 receptor 1 localized to the MEJ15 and induces an elevation of EC [Ca2+], after UV uncaging is plotted at 0, 1, and 20 minutes after PE stimulation. In H and I, Vasoconstriction response measuring percentage change of initial diameter to PE in TD arteries pretreated with C3 inhibitor and then stimulated with PE for 0, 1, 5, 10, and 20 minutes. Data are represented as the mean±SEM. (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.

localize to the MEJ, thereby providing an optimal microsignaling domain whereby binding partners, including Cx43, eNOS, and many other proteins, could cluster.10–17,35,40 Indeed, spatial partitioning of proteins within a cell provides an important level of control to ensure fidelity of cell signaling. Accumulating evidence from monolayers of cultured cells has suggested that localized eNOS could allow for NO to be generated in a specific cellular region.22,23 It is reasonable to speculate that the active pool of eNOS we observe at the MEJ is regulating basal vascular tone and blood pressure because we show, with L-NAME, induced constriction in Figure 2. This would provide an energy-efficient mechanism to minimize NO diffusion distance to the SMCs. Thus, our identification of a pool of compartmentalized active eNOS uniquely at the MEJ throughout different vascular beds places these initial descriptive observations into a physiological context.

Our data go beyond the possibility of paracrine release of NO at the MEJ mediating the magnitude of vasoconstriction and suggest another function for the pool of localized eNOS
at the MEJ (i.e., 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, whereas other reports 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. 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 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, thioredoxin-1, and carboxyl reductase, 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 Ca2+/H1-dependent signaling pathway based on the rapid IP3-induced increase in EC [Ca2+]i 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 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 vaso-motor tone and blood pressure, (2) local S-nitrosylation of

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**Figure 6.** Schematic summary of Cx43 S-nitrosylation/denitrosylation regulating heterocellular communication in the vessel wall. Application of PE stimulates the α1 receptor (1), followed by the induction of IP3 release in SMCs (2). The release of IP3 activates intracellular calcium stores in endoplasmic reticulum promoting SMC contraction (3). In addition, IP3 traverses S-nitrosylated Cx43 GJ channels at the MEJ to stimulate IP3 receptors in ECs, inducing calcium release (5). GSNOR activity increases (6), which promotes denitrosylation of Cx43, altering channel permeability (7). Calcium and phosphorylation activate eNOS, resulting in released NO (8) to promote SMC relaxation and renitrosylation of Cx43 to open GJ channels (9).
Cx43 to regulate more permeable channels, and (3) local production of NO for immediate feedback on SMCs. The presence of GSNOR provides a check on this system by inducing Cx43 denitrosylation on constriction and inducing a less permeable GJ. The results provide an investigative framework for future endeavors focusing on the eNOS/GSNOR axis as a potential therapeutic target for treating vascular pathological features, such as hypertension.

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Disclosures

None.

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