Rapid Endothelial Cell–Selective Loading of Connexin 40 Antibody Blocks Endothelium-Derived Hyperpolarizing Factor Dilation in Rat Small Mesenteric Arteries

Simon Mather, Kim A. Dora, Shaun L. Sandow, Polly Winter, Christopher J. Garland

Abstract—In resistance arteries, spread of hyperpolarization from the endothelium to the adjacent smooth muscle is suggested to be a crucial component of dilation resulting from endothelium-derived hyperpolarizing factor (EDHF). To probe the role of endothelial gap junctions in EDHF-mediated dilation, we developed a method, which was originally used to load membrane impermeant molecules into cells in culture, to load connexin (Cx)-specific inhibitory molecules rapidly (≈15 minutes) into endothelial cells within isolated, pressurized mesenteric arteries of the rat. Validation was achieved by luminally loading cell-impermeant fluorescent dyes selectively into virtually all the arterial endothelial cells, without affecting either tissue morphology or function. The endothelial monolayer served as an effective barrier, preventing macromolecules from entering the underlying smooth muscle cells. Using this technique, endothelial cell loading either with antibodies to the intracellular carboxyl-terminal region of Cx40 (residues 340 to 358) or mimetic peptide for the cytoplasmic loop (Cx40; residues 130 to 140) each markedly depressed EDHF-mediated dilation. In contrast, multiple antibodies directed against different intracellular regions of Cx37 and Cx43, and mimetic peptide for the intracellular loop region of Cx37, were each without effect. Furthermore, simultaneous intra- and extraluminal incubation of pressurized arteries with inhibitory peptides targeted against extracellular regions of endothelial cell Cxs (Gap 26, Gap 27, and Gap 27; 300 μmol/L each) for 2 hours also failed to modify the EDHF response. High-resolution immunohistochemistry localized Cx40 to the end of endothelial cell projections at myoendothelial gap junctions. These data directly demonstrate a critical role for Cx40 in EDHF-mediated dilation of rat mesenteric arteries.

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Key Words: endothelium-derived hyperpolarizing factor □ myoendothelial gap junctions □ endothelium-dependent dilation □ acetylcholine □ connexin 40

In arterioles and some arteries, gap junctions between endothelial and smooth muscle cells (myoendothelial gap junctions [MEGJs]) enable changes in membrane potential to spread over considerable distances and, as a consequence, regulate blood flow by coordinating diameter change through the microcirculation.1–3 For example, injection of hyperpolarizing current into a single endothelial cell can evoke extensive relaxation involving many smooth muscle cells throughout an isolated arteriole.4,5 An important aspect of this response is that MEGJs may provide a crucial route for endothelial cell hyperpolarization to spread radially to the adjacent smooth muscle and evoke the dilation attributed to endothelium-derived hyperpolarizing factor (EDHF).6–8 In many arteries, there is more than 1 underlying mechanism for endothelium-dependent hyperpolarization of smooth muscle cells. In the rat mesenteric artery, during submaximal contraction to phenylephrine (PE), EDHF dilation appears to reflect hyperpolarizing current spread through MEGJs and an action of endothelium-derived K+.9,10 However, with maximal PE contraction, the K+ component is blocked; therefore, the unidentified mechanism predominates.11

Although ultrastructural studies have demonstrated the presence of MEGJs in a number of resistance arteries, these structures do not appear to be ubiquitous.12 Furthermore, when MEGJs are present, showing that they are both patent and necessary for an aspect of smooth muscle cell function has not proved to be straightforward. Studies that have attempted to correlate the heterocellular spread of dyes and current have been confounded by a dissociation between these parameters, while the use of gap junction blockers, such as glycyrrhetinic acid and its derivatives, have been complicated by the variety of indirect effects exerted by these agents on vascular cells.13–15 Synthetic peptides with homology to conserved extracellular regions of vascular connexins (Cx) have been suggested to provide selective block.7 However, how these agents might block gap
junctions is not known, and the possibility that they may exert indirect effects has not been satisfactorily excluded. In spite of these persistent concerns, experiments using combinations of Cx-mimetic peptides in rabbit iliac artery have suggested a role for Cx37 and Cx40 in the radial spread of endothelial hyperpolarization.\(^\text{16}\) This contrasts with studies in another large vessel, the porcine coronary artery. Gap peptides failed to modify muscle hyperpolarization immediately below the internal elastic lamina, but spread through the media was markedly reduced, suggesting the peptides blocked homocellular smooth muscle gap junctions, rather than MEGJs.\(^\text{17}\)

The potential to target MEGJs directly would be increased considerably were it possible to load putative blockers selectively into the endothelium of intact vessels, minimizing simultaneous effects within the smooth muscle layers. Recent work suggests that a method originally used with cultured cells\(^\text{18}\) may enable rapid and selective loading of molecules into endothelial cells in situ. By altering osmolarity using a commercially available kit, macromolecules were introduced into endothelial cells in rabbit resistance arteries.\(^\text{19}\) However, commercially available kit, macromolecules were introduced into endothelial cells in situ. By altering osmolarity using a commercially available kit, macromolecules were introduced into endothelial cells in rabbit resistance arteries.\(^\text{19}\) However, in extensive preliminary experiments using a similar kit, we were unable to maintain resistance arteries (diameter, \(\approx350\) \(\mu\)m) in a viable state; therefore, we modified the original method for loading cells in culture for use in isolated resistance-size arteries under physiological pressures.

We validated the technique using electron and fluorescence microscopy and using functional studies to show that EDHF-mediated dilation in rat pressurized mesenteric arteries maximally stimulated with PE can be significantly disrupted with antibodies and mimetic peptides directed against intracellular regions of Cx40 but not Cx37 or Cx43.

**Materials and Methods**

**Measurement of Diameter and Endothelial Cell Calcium in Pressurized Arteries**

Male Wistar rats (Charles River; 200 to 250 g) were anesthetized and euthanized following procedures defined under the UK Animals (Scientific Procedures) Act 1986. Segments (2- to 3-mm long) of a third-order branch (ID, \(\approx250\) to 350 \(\mu\)m) of superior mesenteric artery were dissected at 4°C and cannulated with glass pipettes (OD, \(\approx150\) \(\mu\)m) in a myograph (Danish Myotechnology 120CP).\(^\text{20}\) To ensure optimal reactivity, the artery was pressurized (50 mm Hg) and stretched longitudinally by 20%.\(^\text{21}\) After 30 minutes superfusion at 37°C, reactivity was assessed by contraction with 3 \(\mu\)mol/L PE, followed by endothelium-dependent relaxation to 1 \(\mu\)mol/L acetylcholine (ACh). Only vessels relaxing \(\approx90\)% of reflecting undamaged endothelium, were subsequently used. Brightfield images (outer diameter) were captured with a laser scanning confocal microscope (FV500-SU, \(\times10\) objective; Olympus) and recorded (Tiempo software, Olympus) at 1 Hz. For Ca\(^2\+) imaging, pressurized arteries were luminal perfused with 3-(N-morpholino) propanesulfonic acid (MOPS) solution containing fluo-4-acetoxymethyl ester (23 \(\mu\)mol/L) and 0.02% Phorbol F-127 selectively to load the endothelial cells.\(^\text{22}\) Fluorescence from endothelial cells at the bottom of the artery (>350 cells) were visualized (512\(\times360\) pixels) and recorded at 2.4 Hz (\(\times20, 0.5\) numerical aperture objective; Olympus). Data were analyzed with Metamorph software (version 6.1; Universal Imaging). The nitric-oxide synthase (NOS) inhibitor \(N\)\(^\circ\)nitro-L-arginine methyl ester (100 \(\mu\)mol/L) was present throughout all experiments.

**Electron Microscopy**

**Conventional**

Pressurized arteries were fixed in 1% paraformaldehyde, 3% glutaraldehyde in 0.1 mol/L sodium cacodylate, with 35 mmol/L betaine, 0.2 mmol/L CaCl, and 0.15 mol/L sucrose (pH 7.4) and processed using standard procedures.\(^\text{12}\) From a section cut perpendicular to the longitudinal axis of each preparation, each of four 15-\(\mu\)m long regions of endothelium 90° apart were photographed at \(\times20\) 000 and vesicle (50- to 200-nm diameter) counts (excluding surface caveolae) made from prints.

**Immunolocalization**

Short segments of artery were isolated from urethane-anesthetized (3 mg/kg) rats, rapidly dissected, and then frozen in liquid nitrogen at \(\approx2100\) bar (BAL TEC, HPM 010). Segments were freeze substituted (Leica, AFS) at -90°C in 0.1% uranyl acetate in acetone over 4 days and embedded in LR gold polymerized under UV light at -25°C. Thin sections were cut and placed on formvar- and carbon-coated slot grids and sequentially incubated in PBS containing 1% normal donkey serum and 0.2% Tween 20 (blocking buffer; 30 minutes) with antibody raised against mouse Cx40 (1:100; Chemicon) in blocking buffer for 18 hours at 37°C and then rinsed and incubated in secondary antibodies conjugated to 5-nm colloidal gold (1:40; British Biocell) in 0.01% Tween 20. Sections were subsequently treated with 1% glutaraldehyde in PBS and stained conventionally.

**Selective Endothelial Cell Loading of Cell-Impermeant Macromolecules**

The effectiveness of macromolecule loading was determined by incorporating fluorescent markers 5(6)-carboxyfluorescein (1 mg/mL), Alexa Fluor 633 goat anti-rabbit IgG (0.2 mg/mL), or fluorescein isothiocyanate (FITC)-conjugated dextran (3 kDa, 2 mg/mL) into the endothelium of pressurized mesenteric arteries. During loading, luminal pressure was reduced to 5 mm Hg. A microperistaltic pump (LKB, Bromma; speed 100 \(\mu\)L/min) was used luminally to perfuse 100 \(\mu\)L of hypertonic solution (800 millimolar [mosM]; 10% polyethylene glycol (PEG) 1000 plus impermeant macromolecule). The solution remained in the lumen for periods specified in Results (10 to 40 minutes) and was then flushed with 200 \(\mu\)L of hypotonic solution (180 mosM, 40% H\(_2\)O added) and remained present for a further 2 minutes, before flushing through 250 \(\mu\)L of isotonic MOPS. After 2 to 10 minutes, arteries were reequilibrated at 50 mm Hg. Endothelial cells were visualized (\(\times20, 0.5\) numerical aperture objective; Olympus) at 5- to 10-minute intervals to determine the extent and stability of loading. The efficiency of loading was estimated to be \(\approx1:1000\) by comparing cellular fluorescence against a standard curve constructed from serial dilutions of carboxyfluorescein in vitro (n=5).

**Drugs and Solutions**

MOPS-buffer of the following composition (mmol/L) was used: 145 NaCl, 4.7 KCl, 2.0 CaCl\(_2\), 2.6 Na\(_2\)HPO\(_4\), 1.17 MgSO\(_4\)-7H\(_2\)O, 2.00 MOPS, 1.2 NaH\(_2\)PO\(_4\)-H\(_2\)O, 5.0 glucose, 2.0 pyruvate (Na salt), 0.02 EDTA free acid, 2.75 NaOH. For the hypertonic solution (800 mosM), sucrose (500 mmol/L), and 10% volume/volume PEG 1000 was added to isotonic MOPS. Hypotonic solution (180 mosM) was 60% MOPS, 40% H\(_2\)O. Drugs were all from Sigma except carboxyfluorescein, FITC-dextran (3 kDa), fluo-4-acetoxymethyl ester, and Fluoronic F-127 (Molecular Probes); PEG 1000 (Calbiochem). Lycocromalim was a gift from GlaxoSmithKline (Stevenage, UK).

Cx40 antibody against amino acids 340 to 358 (carboxyl-terminus; Chemicon); Cx37 antibody against amino acids 318 to 333 (carboxyl-terminus; Alpha Diagnostics) and 99 to 147 (cytoplasmic loop; Diathema); Cx43 antibody against amino acids 363 to 382 and 130 to 143 (carboxyl-terminus and cytoplasmic loop; both from Sigma). These antibodies have been previously characterized in both transfected Rin and HeLa cells and arterial tissue.\(^\text{23,24}\) Antibodies were diluted (1:10) to give a luminal concentration of 50 \(\mu\)g/mL. All stock solutions were prepared using distilled water.

Cx-mimetic peptides \(^{46}\)Gap 26 (VCYDKSPFSPHVR), \(^{46}\)Gap 27 (SRPTEKVNVFIV), and \(^{46}\)Gap 27 (SRPTEKTIFFI) with homology to the first (Gap 26) and second (Gap 27) extracellular Cx-loops were made from prints.

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loading, peptides with homology to the cytoplasmic loop amino acids 130 to 140 of rCx37 (EHQMAKISVAE) and mCx40 (AELSC-WKEVDG) were obtained from Auspep (Parkville, Australia; ≥95% purity). Peptides targeting this region have been used previously as active loading of the smooth muscle (Figure 1B). Abluminal creasing intraluminal exposure to 20 minutes caused substantive loading with carboxyfluorescein (Figure 1A). In- 

A 10-minute hypertonic exposure was optimal for selective loading of the smooth muscle (Figure 1B). Abluminal increasing luminal exposure to 40 minutes did not load the smooth muscle with these larger molecular-mass dyes, apart from the occasional cell. 

After endothelial loading with carboxyfluorescein (for 10 minutes), subsequent images at 5-minute intervals revealed gradual loss of fluorescence (Figure 1B). By 30 minutes, most dye fluorescence was gone. This did not appear to reflect bleaching, as similar loss was apparent if fluorescence was assessed only once, at 30 minutes postloading. Following the loss of carboxyfluorescein fluorescence from the endothelium, the same cells could be "reloaded" with dye by simply repeating the osmotic loading protocol, with vascular reactivity retained. As IgG or dextran loading gave a stable fluorescent signal over time, carboxyfluorescein may be actively extruded by the cells.

Statistical Analysis

Peak increases in outer diameter at each concentration of ACh were used to construct cumulative concentration-response curves (10 to 3 μmol/L) and ECsubscript 50 derived using a curve-fitting program (Prism 4.0). All data are means±SEM of n replicates, with n representing individual rats. Fluorescence data are summarized as average fluorescence from 16 randomly chosen endothelial cells (F), sampled at 5-second intervals relative to basal intensity (Fsubscript 0). Analysis used either paired Student t test or 1-way ANOVA with Bonferroni post test and rejection of the null hypothesis at P<0.05.

Results

Hyperosmolality Increases Endothelial Cell Pinocytic Vesicles Without Changing Cell Integrity

Pinocytic loading solutions did not alter the ultrastructural integrity of the endothelium (online Figure I in the data supplement available at http://circres.ahajournals.org). Tissue fixed in hypertonic loading solution showed an ≈4-fold increase in intracellular vesicles within endothelial cells (online Figure I) and a reduction in cell volume. In contrast, after completing the loading protocol, the number of vesicles and the cell volume returned to control levels. Neither treatment altered ultrastructural morphology in the adjacent smooth muscle cells. Furthermore, the loading protocol damaged <5% of endothelial cells, assessed by staining dead cells with propidium iodide (online Figure IIIA).

Changing Luminal Osmolarity Selectively Loads Endothelial Cells With Fluorescent Markers

A 10-minute hypertonic exposure was optimal for selective cellular loading with carboxyfluorescein (Figure 1A). Increasing intraluminal exposure to 20 minutes caused substantive loading of the smooth muscle (Figure 1B). Abluminal exposure of pressurized arteries to similar solutions directly loaded the smooth muscle and perivascular nerves with carboxyfluorescein (online Figure II A). Selective, extensive endothelial cell loading also followed 10-minute luminal incubation with hypertonic solution containing Alexa Fluor 633 IgG or FITC-labeled dextran and without significant smooth muscle loading (Figure 1A). Unlike carboxyfluorescein, increasing luminal exposure to 40 minutes did not load the smooth muscle with these larger molecular-mass dyes, apart from the occasional cell.

After endothelial loading with carboxyfluorescein (for 10 minutes), subsequent images at 5-minute intervals revealed gradual loss of fluorescence (Figure 1B). By 30 minutes, most dye fluorescence was gone. This did not appear to reflect bleaching, as similar loss was apparent if fluorescence was assessed only once, at 30 minutes postloading. Following the loss of carboxyfluorescein fluorescence from the endothelium, the same cells could be "reloaded" with dye by simply repeating the osmotic loading protocol, with vascular reactivity retained. As IgG or dextran loading gave a stable fluorescent signal over time, carboxyfluorescein may be actively extruded by the cells.

Changing Luminal Osmolarity Does Not Alter Functionality in Pressurized Arteries

Endothelium-dependent relaxation to ACh (10 nmol/L to 3 μmol/L; ECsubscript 50, 177 nmol/L; maximal relaxation [Rmax], 93±3%; n=8) was not altered by the osmotic loading protocol (ECsubscript 50, 170 nmol/L; Rmax, 90±2%; n=8; Figure 2A and 2B). Likewise, neither the initial contraction to 3 μmol/L PE (77.4±1.9%; n=13) nor dilation to 3 μmol/L levcromakalim (90.3±1.9%, n=12) or 15 μmol/L cyclopiazonic acid (84.2±3.3%, n=13) was altered.

As an increase in endothelial cell [Ca2+]i to ACh is critical for endothelial and subsequent smooth muscle cell hyperpolarization, endothelial cell viability was assessed as time course of rises in [Ca2+]i, in individual endothelial cells in pressurized arteries (Figure 2C and 2D). Before and after the
osmotic loading protocol, ACh stimulated sustained and oscillating rises in endothelial cell \([Ca^{2+}]_i\) (Figure 2C), the oscillations remaining asynchronous between cells, with repetitive waves of \([Ca^{2+}]_i\) moving longitudinally across endothelial cells. Therefore, the loading protocol alone did not modify the ability of the endothelium to increase \([Ca^{2+}]_i\) and evoke EDHF responses.

**EDHF-Mediated Dilatation to ACh: Effect of Endothelial Cell Loading With Cx Antibodies and Mimetic Peptides**

Endothelial cell loading with antibodies directed against the intracellular carboxy-terminus of Cx40 did not alter EDHF-mediated dilation in arteries submaximally contracted to 0.9 \(\mu\text{mol/L}\) PE (control: \(EC_{50}\) 130 \(\mu\text{mol/L}\); \(R_{\text{max}}\) 97.0±0.8%; after antibody: \(EC_{50}\) 99 \(\mu\text{mol/L}\); \(R_{\text{max}}\) 97.6±0.8%; \(n=4\); Figure 3A and 3B) or in the additional presence of ouabain (online Figure IIIA). In marked contrast, raising the concentration of PE to evoke maximal contraction (and inhibit the simultaneous influence of diffusible EDHF apparent during submaximal stimulation\(^{11,20}\)) was associated with block of EDHF dilation (Figure 3A and 3B and online Figure IIIA). In vessels contracted with 5 \(\mu\text{mol/L}\) PE, the \(R_{\text{max}}\) was only 26.7±8.8% (\(n=11\)), whereas subsequent addition of 3 \(\mu\text{mol/L}\) levocromakalim evoked complete dilation (96.9±0.9%; \(n=4\); Figure 3A). Cx40 antibody did not alter either the increase or oscillations in endothelial cell \([Ca^{2+}]_i\) to ACh despite block of dilation (Figure 3C), and staining with propidium iodide indicated damage to <8% of cells. In contrast, endothelial loading with antibodies against both the carboxy-terminus and the cytoplasmic loop of Cx37 and Cx43 failed to modify EDHF dilation significantly (Figure 4A and 4B). Furthermore, if Cx40 antibody was loaded before block of NOS, dilation was sustained until \(N^\text{G}-\text{nitro-L-arginine methyl ester}\) was applied (online Figure IIIB).

Overall, this profile was mirrored by endothelial cell loading with intracellular Cx-mimetic peptides. Cx40 peptide suppressed EDHF dilation in arteries contracted with 5 \(\mu\text{mol/L}\) PE, whereas the Cx37 peptide had no significant effect (Figure 4C).

**Effect of Extracellular Cx-Mimetic Peptides and Carbenoxolone Against EDHF Dilation**

Simultaneous intra- and extraluminal incubation of pressurized arteries (for 2 hours; 3×40 minutes exposures) with a combination of \(^{43}\text{Gap 26}, \^{40}\text{Gap 27}, \text{and } \^{37,43}\text{Gap 27}\) (each 300 \(\mu\text{mol/L}\)) failed to modify EDHF dilation to ACh (Figure 5A). In contrast, whereas the gap junction blocker carbenoxolone (100 \(\mu\text{mol/L}\)) did not alter the amplitude of, or oscillations in, endothelial increases in \([Ca^{2+}]_i\) (Figure 5C and 5D), it completely blocked EDHF dilation (Figure 5B).

**Ultrastructural Localization of Cx40**

Cx40 was localized to points of close association between endothelial and smooth muscle cells that also exhibited small regions of pentalaminar membrane, characteristic of MEGJs (Figure 6).
There are 2 major observations in this study. First, we show that it is possible to rapidly and selectively load large membrane impermeant molecules, including antibodies, into the endothelium of arteries under physiological pressures, without disrupting function. Second, using this approach combined with high-resolution ultrastructural methods, we provide direct evidence that MEGJs play a central role in EDHF dilation and highlight a primary role for Cx40 in the radial transfer of this signal from the endothelium.

The basis for cell loading is the normal process of endocytosis, describing the nondisruptive passage of macromolecules across the plasma membrane by phagocytosis or “cell eating” and pinocytosis or “cell drinking.”

Figure 3. Effect of endothelial cell Cx40 antibody loading on EDHF dilation and endothelial cell [Ca^{2+}] increase. A, Time course of diameter following application of ACh in an artery before and after luminal loading Cx40 antibody (Ab). When the artery was contracted with 5 μmol/L (High) (but not 0.9 μmol/L in the same artery [Low]) PE, the dilation to ACh was fully blocked. B, Summarized data are from 4 to 6 paired experiments. C, Time course of changes in endothelial cell [Ca^{2+}] (F/F_o) in response to ACh (0.3 μmol/L, added at 0) in arteries where pinocytic loading of Cx40 antibody fully blocked functional responses. Thin lines represent average cellular fluorescence intensity in individual cells; bold line, average of 16 cells. Summary data represent mean responses to 0.3 μmol/L ACh added at t=0 s (n=4 experiments; control taken from Figure 2, postloading).

Figure 4. Effect of endothelial cell loading of connexin-specific inhibitory molecules on EDHF dilation in pressurized 12.6 pd arteries. Antibodies against Cx37 (A) (n=4) and Cx43 (B) (n=3) or intracellular Cx37 mimic peptide (C) (n=3) did not alter EDHF dilation in arteries maximally stimulated with 5 μmol/L PE, whereas Cx40 peptide blocked dilation (C, n=4). All paired experiments.

Discussion

There are 2 major observations in this study. First, we show that it is possible to rapidly and selectively load large membrane impermeant molecules, including antibodies, into the endothelium of arteries under physiological pressures, without disrupting function. Second, using this approach combined with high-resolution ultrastructural methods, we provide direct evidence that MEGJs play a central role in EDHF dilation and highlight a primary role for Cx40 in the radial transfer of this signal from the endothelium.

The basis for cell loading is the normal process of endocytosis, describing the nondisruptive passage of macromolecules across the plasma membrane by phagocytosis or “cell eating” and pinocytosis or “cell drinking.” Only a few specialized cells, such as macrophages and neutrophils, use phagocytosis, whereas virtually all cells can carry out some

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form of pinocytosis. An ability to increase the formation of pinocytic vesicles in cells was first described in L929 cells. Vesicles were then ruptured (by altering the osmolarity), enabling intracellular release of horseradish peroxidase, anti-ricin antibodies, or dextran (70 kDa), an effect termed osmotic lysis of pinosomes. A similar approach in whole tissue (rabbit mesenteric artery) used a commercially available kit, but, although we could load rat mesenteric smooth muscle cells with fluorescently-labeled dextran using kit reagents, endothelium-dependent vasodilation was lost after only 5 minutes of exposure. We, therefore, developed the original protocol for use in pressurized arteries.

Selective endothelial cell incorporation of macromolecules was facilitated by using artery segments cannulated at both ends. Luminal solution was changed independently of the bathing solution, and a cycle of 10 minutes hypertonic, 2 minutes hypotonic, before returning to isosmotic conditions, proved optimal to load the endothelium with fluorescent markers of differing structure and size: carboxyfluorescein, FITC-dextran, and Alexa Fluor 633 IgG. If the hyperosmotic phase was prolonged, carboxyfluorescein appeared in the smooth muscle, which might reflect movement through the myoendothelial gap junctions present in this artery. Molecules up to ~1 kDa can pass through gap junctions; therefore, this seems a likely explanation for carboxyfluorescein (376 Da), supported by the observation that equivalent muscle loading was not evident after endothelial cell loading with either IgG (~150 kDa) or dextran (3 kDa). In contrast to these markers, carboxyfluorescein-fluorescence within the endothelium decreased rapidly, presumably reflecting, in part, the activity of ATP-binding cassette transporters within the endothelial membrane, which extrude a range of molecules, including carboxyfluorescein and calcine.

Following technique validation, the role of MEGJs in EDHF dilation was probed. Agonist activation of endothelial cells in the mesenteric artery increases [Ca²⁺] and stimulates hyperpolarization, followed by hyperpolarization and relaxation in the adjacent smooth muscle. That radial heterocellular coupling is important in local, endothelium-driven dilation is supported in the rat mesenteric artery by the presence of heterocellular membrane associations of pentalamellar structure, characteristic of MEGJs. However, although rat mesenteric artery endothelial cells express Cx37, Cx40, and possibly Cx43 (but not Cx45), the precise profile in the smooth muscle is not clear. Thus, it is not known which Cxs are expressed at the MEGJs.

Endothelial cell hyperpolarization reflects the opening of Ca²⁺-activated K⁺ channels amplified by K⁺ efflux through the inwardly rectifying K⁺ channels focused within the endothelium of the mesenteric artery. Extracellular K⁺ accumulation then stimulates smooth muscle hyperpolarization and relaxation by activating Na⁺/K⁺-ATPase. However, if background contraction with PE is increased, K⁺ loses the ability to serve as an EDHF, whereas smooth muscle cell hyperpolarization and relaxation to ACh is sustained by a persistent parallel mechanism. Loss of K⁺ as an EDHF appears to be caused by BKCa-channel activation in the smooth muscle (by PE-mediated depolarization and rises in [Ca²⁺]), with consequent K⁺ efflux eventually max-
mally activating the Na⁺/K⁺-ATPase), as it is reversed in the presence of the BK<sub>ca</sub> blocker iberiotoxin. In the present study, possible input from K⁺ as an EDHF was blocked by high levels of background arterial contraction to 5 μmol/L PE, isolating EDHF dilation, which may be suggested to be attributable to MEGJs in this artery.

The present study provides direct evidence that the MEGJs mediate the persistent dilation. Endothelial cell loading with antibody to the intracellular carboxy-terminal region of Cx40 completely abolished EDHF dilation, without altering the increase in [Ca<sup>2+</sup>], necessary to initiate hyperpolarization, when arteries were stimulated with higher but not lower concentrations of PE. In these experiments, direct muscle dilation to levocromakalim persisted, and in separate experiments, EDHF dilation was maintained after endothelial loading with inhibitory molecules against different intracellular regions of Cx37 or Cx43. Interestingly, when arteries were contracted with low concentrations of PE, Cx40 antibody-insensitive ACh relaxation appeared not solely attributable to release of K⁺ acting on smooth muscle Na⁺/K⁺-ATPase. Although ouabain alone shifted the ACh-dilation curve, it did not abolish dilation in the presence of Cx40 antibody. This suggests compensatory pathways can still operate under low tone, possibly via electrical coupling through MEGJs comprised of Cx37.

A role for Cx40 in heterocellular signaling is supported by ultrastructural localization of this Cx to the end of endothelial-derived projections associated with MEGJs. There is only 1 other equivalent ultrastructural report, localizing Cx40 within the membrane of gap junctions between endothelial cells and smooth muscle cells modified to secrete renin in the terminal region of kidney afferent arterioles. Whether or not this Cx is the primary Cx for myoendothelial signaling remains to be seen.

The Cx-mimetic peptides loaded into the endothelium were homologous to segments of the intracellular cytoplasmic loop sequences (amino acids 130 to 140) of Cx37 and Cx40. Patch-clamp experiments suggest this region may represent a Cx-gating element, making it a potentially useful target, particularly because there is no sequence homology here between Cx37 and Cx40. An ability to block EDHF dilation with Cx40 intracellular-mimetic peptide contrasts with the lack of effect of prolonged extracellular application of a triple combination of Gap peptides targeting extracellular Cx regions. This triple combination has been suggested as the most effective and reliable way to block vascular gap junctions. Previous use of Gap peptides against EDHF responses in the mesenteric artery provide inconsistent data. Gap 27 failed significantly to reduce EDHF hyperpolarization in 1 study, reduced it by ~65% in another, and almost abolished EDHF dilation in pressurized mesenteric arteries. Interestingly, in the latter, Gap peptide was applied via the lumen of pressurized arteries, so it would not be predicted to even reach the MEGJs, due to the tight junctions between the endothelial cells. In any event, the reason such discrepancies exist is not clear. One possibility may be an alteration in pH caused by the peptides. In our experiments, where the triple peptide combination was applied within the lumen and around the artery at the same time, the reduction in pH caused by the

Figure 6. Cellular localization of Cx40. Cx40 antibody conjugated to 5-nm colloidal gold was localized to small regions at the end of the endothelial cell (ec) projections passing through the internal elastic lamina (iel) and contacting the adjacent smooth muscle cell (smc) (A, inset). These sites are analogous to the MEGJs in conventional ultrastructural studies (B, inset). Small regions of pentalaminar membrane, characteristic of gap junctions (A and B, insets; C and D) are close to the gold label (A and C, insets). Conventional fluorescence immunohistochemistry (flattened segments, Cx40-conjugated Alexa-633 visualized in combined 160 nm optical sections) localized Cx40 to adjacent endothelial cell borders (E). Holes in the internal elastic lamina (autofluorescence; F, arrows) are potential sites for MEGJs to pass through. Overlay of a single confocal section (at internal elastic lamina-smooth muscle cell interface) of the internal elastic lamina at the same site reveals bright spots of fluorescence, indicating potential MEGJ Cx40 labeling (G, arrows), whereas endothelial cell border staining is limited (eg, F and G). Arrows in E through G are at the same site in each panel and highlight the close spatial relationship of MEGJs and endothelial cell-endothelial cell gap junctions. Bars in A and B, 1 μm; A inset and D, 50 nm; B inset and C, 100 nm; C inset, 25 nm; E through G, 25 μm.
peptides was corrected. Lack of effect of Gap peptides against EDHF dilation is similar to the lack of effect of 37,40 Gap 26 in mouse mesenteric arteries, where MEGJs are abundant.47

Data linking cell-specific Cx isoforms to functional responses in vascular tissue are limited. A major physiological role for Cx40 is indicated by studies with knockout (Cx40−/−) mice. These mice are hypertensive, and, even without NOS block, cremaster arteriolar dilation to 10 μmol/L ACh was attenuated.48 Furthermore, the ability of arterioles to conduct vasodilatation longitudinally through the vessel wall was markedly attenuated in these animals.48,49 A response normally ascribed to the endothelium.3,50 However, it remains possible that long-term compensatory mechanisms may underlie these changes in genetically modified mice, whereas short-term block in the present study circumvents this potential problem. The only equivalent long-term data come from a recent study with Gap peptides in the rabbit iliac artery (≈10 smooth muscle layers), suggesting both Cx37 and Cx40 are essential for hyperpolarization to spread through MEGJs.16 However, in this particular artery, definitive identification of MEGJs, and the constitutive Cxs, is required.

In summary, by selectively loading inhibitory Cx antibodies and peptides into endothelial cells in pressurized rat mesenteric arteries, we have demonstrated directly a major functional role for the MEGJs known to occur in this artery. Furthermore, our data suggest that Cx40 is the critical Cx isoform involved in heterocellular signaling.

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References


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Supplementary Figure 1  Effect of luminal pinocytic loading protocol on arterial ultrastructure. (A-E) Electron micrographs of radial cross-sections through isolated, pressurized arteries fixed before (A, B (top left), C), during (B (top right), D) and after fully completing (B (lower), E) the pinocytic loading protocol. Cellular integrity was maintained throughout (A-E). Compared to control (C), the hypertonic treated tissue (D) showed an ~4-fold increase in the number of intracellular endothelial vesicles (excluding surface caveolae), and a reduction in endothelial cell volume. After the completed protocol, the number of such vesicles decreased to control levels (E) and endothelial cell volume was the same as control. Neither the hypertonic solution nor the full protocol had any effect on adjacent smooth muscle cell morphology (B) or the presence of close myoendothelial associations (D, E; upper right panels). Strikingly, the integrity of gap junctions between adjacent endothelial cells was disrupted during hypertonic treatment, but returned to that of control after the full protocol (D, E; lower right panels between arrows). (A) Bar, 100 μm. (B) Bar, 10 μm. (C-E) Left main panels, top right panels, bar 1 μm; insets 200 nm. (D, E) bottom right panels, bar 50 nm. iel, internal elastic lamina. Representative images from 3 arteries per protocol.
**Supplementary Figure 2**  Assessment of cellular damage and pinocytic loading of nerves and smooth muscle cells. (A) Inverted images of propidium iodide (0.01 %) staining of endothelial cells following damage to all endothelial cells (includes autofluorescence overlay) compared to the luminal pinocytic loading protocol, boxes 150 µm. (B) Confocal fluorescent images of isolated, pressurized arteries showing intracellular carboxyfluorescein in either perivascular nerves (PVN, bar 50 µm) or smooth muscle cells (SMC, bar 100 µm) following abluminal pinocytic loading. The longitudinal axis of the artery is horizontal in all images.
**Supplementary Figure 3**

**A** Effect of endothelial cell Cx40 antibody loading on endothelium-dependent dilation to increasing concentrations of ACh in pressurized mesenteric arteries stimulated with 1 µmol/L PE (Low) or with 5 µmol/L PE (High). Loading Cx40 in the presence of 100 µmol/L ouabain shifted the ACh-dilation curve only slightly to the right (n = 4) compared to the effect of ouabain alone. Subsequently, the same arteries were contracted with 5 µmol/L PE and now dilation to ACh was effectively blocked. 100 µmol/L L-NAME was present throughout each experiment.

**B** Effect of endothelial cell Cx40 antibody loading on endothelium-dependent dilation to increasing concentrations of ACh in pressurized mesenteric arteries stimulated with 5 µmol/L PE. In the absence of L-NAME, loading endothelial cells with Cx40 Ab (n = 4) shifted the ACh-dilation curve (n = 9) to a similar extent as the combined application of 50 nmol/L apamin, to block SKCa, and 1 µmol/L TRAM-34 to block IKCa (n = 4). The subsequent addition of 100 µmol/L L-NAME (to block nitric oxide release) effectively abolished dilation in Cx40 Ab loaded arteries (data included in Fig. 3B) and arteries exposed to apamin and TRAM-34 (n = 4). At the end of each experiment, propidium iodide (0.01 %) staining indicated damage to <5% of endothelial cells.
Supplementary Figure 1
Supplementary Figure 2

A  EC-selective damage

Pinocytic loading protocol

B  Carboxyfluorescein (PVN)

Carboxyfluorescein (SMC)
Supplementary Figure 3

A

- L-NAME
- L-NAME + Ouabain
- L-NAME + Ouabain + Cx40 Ab Low
- L-NAME + Ouabain + Cx40 Ab High

B

- Control
- Cx40 Ab
- Apamin + TRAM-34
- L-NAME + Apamin + TRAM-34

% Dilution vs log ACh [M]