Flow regulates intercellular communication in HAEC by assembling functional Cx40 and Cx37 gap junctional channels

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Ebong, Eno Essien, Sanghee Kim, and Natacha DePaola. Flow regulates intercellular communication in HAEC by assembling functional Cx40 and Cx37 gap junctional channels. Am J Physiol Heart Circ Physiol 290: H2015–H2023, 2006. First published December 16, 2005; doi:10.1152/ajpheart.00204.2005.—Direct cell-to-cell transfer of ions and small signaling molecules via gap junctions plays a key role in vessel wall homeostasis. Vascular endothelial gap junctional channels are formed by the connexin (Cx) proteins Cx37, Cx40, and Cx43. The mechanisms regulating connexin expression and assembly into functional channels have not been fully identified. We investigated the dynamic regulation of endothelial gap junctional intercellular communication (GJIC) by fluid flow and the participation of each vascular connexin in functional human endothelial gap junctions in vitro. Human aortic endothelial cells (HAEC) were exposed for 5, 16, and 24 h to physiological flows in a parallel-plate flow chamber. Connexin protein expression and localization were evaluated by immunocytochemistry, and functional GJIC was evaluated by dye injection. Connexin-mimetic peptide inhibitors were used to assess the specific connexin composition of functional channels. HAEC monolayers in culture exhibited baseline functional communication at a striking low level despite abundant expression of Cx43 and Cx40 localized at cell-to-cell appositions. Upon exposure to flow, GJIC by dye spread demonstrated a significant time-dependent increase from baseline levels, reaching 7.5-fold in 24 h. Inhibition studies revealed that this response was mediated primarily by Cx40, with lesser baseline levels, reaching 7.5-fold in 24 h. Inhibition studies revealed that this response was mediated primarily by Cx40, with lesser contributions of the other two vascular connexins assembled into functional homotypic and/or heterotypic channels. This is the first study to demonstrate that flow simultaneously and differentially regulates expression of the Cx37, Cx40, and Cx43 proteins and their involvement in the augmentation of intercellular communication by dye transfer in human endothelial cells in vitro.

cell-cell communication; connexins; gap junctions; endothelial cells; fluid shear

INTERCELLULAR COMMUNICATION is a key regulator of vascular function (23, 40). In the vessel wall, cell-to-cell communication occurs by extracellular diffusion and convection of humoral factors or by intercytoplasmic exchange of ions, metabolites, and small signaling molecules (<1 kDa) via gap junctions. Endothelial gap junctions are channels that permit and strictly regulate communication throughout the endothelial monolayer and between endothelial cells and adjacent smooth muscle and circulating blood cells. Endothelial cell migration and growth, particularly following injury and during angiogenesis, depend on communication through gap junctions (31, 33, 38, 51, 52). In addition, gap junctions coordinate vascular tone and vasomotion (11, 13, 17) and participate in the regulation of immunoinflammatory responses (36, 50).

Gap junctions are formed by a pair of hemichannels called connexons, each contributed by one of two neighboring cells. Connexons are composed of six connexin monomer subunits arranged around a central pore. The connexins belong to a multigene family of at least 20 related proteins that have been identified in mice and humans. Connexin 37, 40, and 43 (Cx37, Cx40, Cx43, respectively) are the major gap junction proteins expressed in vascular endothelial cells (7, 32, 34, 39, 45). These proteins are very dynamic, exhibiting rapid turnover times and variable expression patterns. Because of the unique gating and permeselective characteristics of Cx37, Cx40, and Cx43, different combinations of these connexin isoforms contribute to homo- or heteromeric connexons and homo- or heterotypic gap junctions leading to a variety of channel types with different functional properties (1, 8, 9, 19, 28, 47–49).

Although the extent of combinations of different connexins within connexons and channels remains unclear, immunohistochemical and immunocytochemical studies demonstrate differential expression and localization patterns of all three vascular connexins in endothelium, depending on species (44), vascular bed (25, 37, 53), and local hemodynamics (21). In vivo studies implicate Cx40 as the constitutive vascular gap junction protein across species and vascular bed, playing an important role in coupling between cells in the vascular wall (44). In addition, studies have demonstrated that Cx40 is homogeneously expressed, along with Cx37, throughout the endothelium (21, 30). In contrast, Cx43 demonstrates to be abundant in vivo only in association with disturbed flow and atherosclerotic lesion-prone regions of vessel bifurcations, where no Cx37 and, in some cases, no Cx40 are found (21, 30). In vitro, it is recognized that cultured endothelial cells abundantly express Cx43 (37, 45), of which its localization, expression, and function can be regulated by fluid flow as demonstrated in experimental studies that replicate physiological flows (15) and hemodynamic features associated with vessel constrictions and bifurcations (16, 21). Thus, of the factors that differentially regulate vascular endothelial connexin expression and localization, the effect of flow associated with different vascular sites is particularly interesting.

We hypothesize that fluid shear stress regulates specific connexin composition and functional state of vascular gap junctions, potentially determining levels and type of communication and possibly cellular phenotype. To test this hypothesis, we investigated gap junctional intercellular communication (GJIC) in human aortic endothelial cells (HAEC) exposed to controlled flows in vitro. We systematically explored the dynamic fluid flow regulation of interendothelial coupling by...
Flow regulation of endothelial gap junctional protein localization and organization was assessed by in situ immunolocalization using the following antibodies: 1) rabbit anti-mouse Cx37 (Alpha Diagnostics, San Antonio, TX), 2) rabbit anti-mouse Cx40 (Chemicon, Temecula, CA), and 3) monoclonal mouse anti-α Cx43 (Chemicon). The specificity of the anti-connexin antibodies used in this study is supported by the manufacturers’ information and published reports by others. The anti-Cx43 is monoclonal made in mouse, specific for Cx43, and recognizes proteins of molecular mass 43–47 kDa (Chemicon). The anti-mouse Cx40 is polyclonal made in rabbit and specific for Cx40 with no significant homology with other connexins (Chemicon). The anti-Cx37 is polyclonal made in rabbit and specific for Cx37 with no significant homology with other connexins (Alpha Diagnostic).

Immediately after flow exposure, experimental and no-flow control monolayers were rinsed in 4°C phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde (5 min), and permeabilized in 0.2% Triton X-100 (2 min) at room temperature. Fixed samples were blocked in PBS containing 3% bovine serum albumin (BSA) and incubated at 4°C overnight with anti-Cx37 antibody (1:10), anti-Cx40 antibody (1:30), or anti-Cx43 monoclonal antibody (1:100), each diluted in PBS containing 3% BSA, followed by incubation in appropriate secondary reagents at room temperature for 30 min. For secondary detection of Cx43, anti-Cx43 was labeled with Alexa Fluor 488 conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:100 in PBS containing 3% BSA. Both anti-Cx37 and anti-Cx43 were detected by the biotin-streptavidin system using biotin-conjugated goat anti-rabbit IgG (1:100) (Zymed, San Francisco, CA) and Alexa Fluor 488 streptavidin (1:100) (Molecular Probes). Specificity of immunolabeling was confirmed by: 1) omission of primary anti-connexin antibodies and incubation of monolayers with secondary reagents only and 2) primary incubation of monolayers with anti-connexin antibodies preadsorbed with the immunogenic peptides against which the antibodies were raised. Samples were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) and viewed by fluorescence microscopy (Olympus IX70), and images were captured using a SensiCam high-performance digital camera and EasyControl software (Cooke, Auburn Hill, MI).

Dye microinjections. Functional GJIC in HAEC monolayers was evaluated by counting the number of dye-coupled cells after single cell injections observed and recorded using fluorescent microscopy and digital imaging. After 5, 16, and 24 h of exposure to flow, samples were transferred from the parallel-plate flow chamber to tissue culture dishes containing fresh, supplemented medium and placed in a 37°C incubator for 0, 30, 60, 90, or 120 min postflow. These monolayers were then relocated to an automatic microscope stage (Prior Scientific, Rockland, MA) at room temperature for single cell intracytoplasmic injections of a dye mixture containing 4% Lucifer yellow (457.3 mol wt) (Molecular Probes) and 1% tetramethylrhodamine dextran (3,000 mol wt) (Molecular Probes) in 0.1 M LiCl-0.05 M Tris, pH 7.8. Lucifer yellow transferred between adjacent cells via gap junction channels. Tetramethylrhodamine dextran, a larger dye that labels the injected cell cytoplasm, only spread across damaged membranes.

With the use of an Eppendorf FemtoJet-Injectman automated microinjection system (Brinkmann, Westbury, NY) at an injection pressure of 700 hPa and an injection time of 1.0 s, six to nine single cells from each slide were selected, and the dye mixture was injected into their cytoplasm. Dye injections on each monolayer were completed within 10 min from the end of the postflow incubation period. Dye transfer after microinjections was observed for 20–30 min by fluorescence microscopy using an Olympus IX70 inverted fluorescence microscope and appropriate filters (Melville, NY). The number of Lucifer yellow-labeled cells surrounding the injected cell was counted 20 min postinjection. Lucifer yellow dye injection and transfer images were captured and recorded using a SensiCam high-performance digital camera and EasyControl software (Cooke). These images were stored and organized using ImagePro (MediaCybernetics, Silver Spring, MD).

Selective inhibition of functional Cx43, Cx40, and Cx37 channels. The extent of Cx43, Cx40, and Cx37 involvement in functional GJIC was investigated by counting the number of dye-coupled cells in the presence of specific mimetic peptide inhibitors. After 5- or 16-h exposure to flow, endothelial monolayers were transferred from the parallel-plate flow chamber to tissue culture dishes containing supplemented fresh medium and placed in a 37°C incubator for either 30, 60, 90, 120, or 150 min postflow. During the latter 30 min of these postflow incubations, after being removed from fresh medium and gently rinsed in fresh PBS, the HAEC monolayers were treated with commercially synthesized connexin-mimetic peptides, including 43Gap26 (VCYDKSFPSHVY), 40Gap27 (SRPT-KNVFYI), and 37,43Gap27 (SRPTEKTIFID) (Sigma Genosys, The Woodlands, TX). The 40Gap26 peptide, specific for Cx40, corresponds to the gap26 domain of the first extracellular loop of Cx43 (6, 41), which differs from Cx37 and Cx40 by just three amino acids (22). 43Gap26 is specific for Cx43 and possesses homology with its gap27 domain of the second extracellular loop (6, 22). 37,43Gap27 is specific for both Cx37 and Cx43 and mimics their second extracellular loop with mimetic peptides, which differs from Cx40 by three amino acids (6, 22).

Inhibitory or toxic effects due to the peptide dissolution components. 40,43Gap26, 40Gap27, and 37,43Gap27, in a nondestructive and reversible manner, rapidly diffuse into gap junction extracellular regions, interrupt interaction between hemichannels, modulate gating, and inhibit dye transfer in channels containing, respectively, either Cx43, Cx40, or both Cx37 and Cx37 channels concurrently (3, 12, 18, 20, 27, 29). The peptides were dissolved in PBS and a minimum volume of either dimethyl sulfoxide (DMSO) or N,N-dimethyl formamide (DMF) and used at a concentration of 250 μM.

For control purposes, a group of HAEC monolayers was not treated with mimetic peptides but was incubated in fresh, supplemented medium and used as a noninhibition reference. Another group of monolayers was treated with PBS containing either 0.2% DMSO or 0.2% DMF, but no mimetic peptides, to verify the absence of inhibitory or toxic effects due to the peptide dissolution components.
After incubation with the specific mimetic peptides, HAEC samples were transferred to fully supplemented growth medium and relocated to the microscope stage at room temperature, and single cells were injected following the same procedure described above for untreated samples. Within 10 min, before the inhibitory effect reversed, the number of dye-coupled adjacent cells was counted to determine the extent to which dye transfer was blocked and to identify the specific connexins responsible for functional GJIC.

**Statistics.** Extents of dye coupling following 0, 5, 16, and 24 h of exposure to flow were normalized values obtained by dividing the counts of dye-coupled cells by the average value of dye coupling in baseline conditions (no-flow or 0-h flow exposure). For all time points examined (0, 5, 16, and 24 h), the normalized values were then averaged and expressed as means ± SE. In examining the effects of specific mimetic peptides on dye coupling compared with untreated flow samples, a Latin square design was used as a statistical method to reduce the residual experimental error by removing variability due to HAEC subculture and postflow incubation time and extents of dye coupling were expressed as means ± SE.

Sets of dye-coupling data (normalized by baseline conditions) were statistically analyzed using either ANOVA or Kruskal-Wallis (non-parametric ANOVA) as appropriate. One-way ANOVA was used as the statistics test for assessing differences between groups confirmed to be normally distributed with zero means and approximately equal variances. One-way ANOVA indicated significant differences between mean values, and the Student-Newman-Keuls test was applied for multiple comparisons for further analysis. Kruskal-Wallis (non-parametric ANOVA) was used for assessing differences between data sets that failed either normality or equal variance tests. The Kruskal-Wallis test indicated significant differences between mean ranks of the raw values. Kruskal-Wallis was followed by Dunn’s method for further analysis. For both ANOVA and Kruskal-Wallis, \( P < 0.05 \) was considered significant unless otherwise specified.

**RESULTS**

**Endothelial baseline intercellular communication in culture.** Immunocytochemical studies confirmed that HAECs used in this investigation express all three vascular connexins. Cx43 is localized primarily at the cell borders with occasional intracellular, nuclear, and perinuclear expression (Fig. 1A). Cx40 was present in cultured endothelial cells and was found at both the cytoplasm and cell borders (Fig. 1B). Cx37 was expressed and primarily associated with the nucleus with insignificant immunoreactivity at the cell cytoplasm and cell borders (Fig. 1C). Control monolayers in which anti-connexin antibodies were omitted or in which each anti-connexin antibody was coincubated with its corresponding immunogenic peptide resulted in greatly reduced immunofluorescence, confirming that the staining observed in the human endothelial monolayers is not artifactual (data not shown).

Cell communication in cultured HAEC monolayers, evaluated by Lucifer yellow dye injection, was strikingly low despite the observed connexin distribution at cell borders. Lucifer yellow dye spread from microinjected endothelial cells was limited to 13.88 ± 1.78 (means ± SE) neighboring cells (Fig. 2).

**Flow-regulated intercellular communication.** A statistically significant increase in the amount of Lucifer yellow transferred to neighboring endothelial cells from single injected cells was demonstrated in monolayers exposed to 11 dyn/cm² laminar flow (Fig. 3). The flow-regulated increase in dye transfer occurred in a time-dependent manner, resulting in 1.8-fold upregulation of dye transfer by 5 h of flow exposure (Figs. 3A and 4) and 3.5-fold upregulation after 16 h of flow (Figs. 3B and 4). Kruskal-Wallis nonparametric ANOVA (with Dunn’s method) was used for assessing significant differences between data sets obtained for various lengths of flow exposure time (Fig. 4), because the data sets failed either normality or equal variance tests.

Immunocytochemical protein localization in endothelial cells exposed to flow for 16 h did not readily reveal any change
in Cx43 immunofluorescence compared with baseline (no-flow control) conditions. The Cx43 protein remained localized in intracellular compartments and was reorganized at cell borders (Fig. 5A). Cx40 protein expression increased with flow and was primarily associated with the nucleus and cell-to-cell appositions (Fig. 5B). Cx37 protein underwent translocation to the perinuclear region (Fig. 5C).

Connexin specificity of functional channels. Inhibition studies with connexin-specific mimetic peptides demonstrated that blocking Cx43 alone or in combination with Cx37 had no effect on Lucifer yellow dye coupling of 5-h flow-conditioned HAEC (Fig. 6). However, a significant 33% reduction in dye transfer was found when Cx40 was blocked (Fig. 6). One-way ANOVA (with Student-Newman-Keuls method) was used as the statistics test for assessing significant differences between data sets obtained for various peptide treatments that followed the 5-h flow, because the data were confirmed to be normally distributed with zero means and approximately equal variances.

After 16 h of flow, blocking of Cx43 still did not reveal any change in flow-induced dye coupling (Figs. 6 and 7, A and B).
Simultaneous blocking of both Cx37 and Cx43 resulted in a statistically significant 26% decrease in dye coupling (Figs. 6 and 7, A and D), and blocking of Cx40 reduced dye coupling by a statistically significant 57% (Figs. 6 and 7, A and C). Kruskal-Wallis nonparametric ANOVA (with Dunn’s method) was used for assessing significant differences between data sets obtained for various peptide treatments that followed the 16-h flow, because the data sets failed either normality or equal variance tests.

No inhibitory or toxic effects were observed when endothelial monolayers were treated with the dissolution solutions of PBS containing either 0.2% DMSO or 0.2% DMF in the absence of mimetic peptides.

**DISCUSSION**

This is the first study to demonstrate that flow augments functional intercellular communication in human endothelial cells in vitro by simultaneous regulation of the three vascular connexins (Cx37, Cx40, and Cx43), each discretely and specifically modulated in their expression, localization, and participation in functional gap junctional communication as evaluated by Lucifer yellow dye transfer. Despite abundant expression of vascular connexin proteins, HAEC in culture revealed a level of communication (assessed by dye transfer) distinctly lower than typically seen in vascular tissue (42). The observed level of cellular coupling may be a consequence of low dye permeability (19), rapid turnover of connexins (4), or lack of full formation and gating of the connexin channels (9). Functional Cx37, Cx40, and Cx43 channels readily transmit Lucifer yellow (19, 35, 43), so it is unlikely that the observed level of dye coupling is due to low Lucifer yellow permeability. It is also unlikely that rapid connexin turnover plays a role in the observed lack of cell-to-cell communication because connexin turnover time in cultured cells (1.5–4 h) (4) is significantly longer than the time required for Lucifer yellow to transfer via gap junction channels (in the order of seconds). Our immunocytochemical studies revealed connexin proteins localized at cell-to-cell appositions in a punctate pattern consistent with the appearance of junctional plaques, yet communication levels were low, leading us to conclude that gap junctional channels were not fully formed from hemichannels or not fully gated open to Lucifer yellow.

Flow-conditioned human endothelial cells exhibited a dramatic increase in cell-to-cell communication, accompanying flow modulation of endothelial connexin expression, localization, and function. These results indicate 1) gating of assembled channels formed by preexisting membranal Cx37, Cx40, and Cx43, and/or 2) trafficking to the cell membrane and assembly into junctional channels of existing cytoplasmic and/or de novo synthesized connexins. The newly functional gap junctional channels could be homotypic or heterotypic. Heterotypic channels may integrate various combinations of the three connexins, with the possible exception of Cx40 together with Cx43. These two connexins have traditionally been reported to be incompatible and unable to form gap junctions permeable by dye or electricity (7, 19, 24). However, in more recent studies there is evidence that Cx40 and Cx43 homomeric and heteromeric connexons may form functional heterotypic channels capable of intercellular dye coupling (14). Our immunocytochemical studies suggest that, given their abundance and localization, Cx40 and Cx43 facilitated by 10.220.33.6 on April 16, 2017 http://ajpheart.physiology.org/ Downloaded from http://ajpheart.physiology.org/ by 10.220.33.6 on April 16, 2017
observed level of dye spread associated with Cx43 may be a result of inefficient channel blocking by the $^{43}\text{Gap26}$ peptide or could be due to the presence of a type of Cx43 function not detectable by Lucifer yellow. $^{43}\text{Gap26}$ inefficiency is unlikely because all peptide inhibitors used in this study ($^{43}\text{Gap26}$, $^{40}\text{Gap27}$, and $^{37,43}\text{Gap27}$) have been demonstrated to efficiently inhibit intercellular dye coupling (3, 12) along with calcium propagation (5, 27) and electrical-coupling via gap junctions (18, 29). In addition, all peptides were applied to endothelial cells for longer than the 15 min required for peptide inhibition to occur (20), and dye injections after peptide washout were completed by 10 min before the inhibitory effect reversed (20). Therefore, we conclude that if there is a flow-mediated attenuation in Cx43 gap junctional communication, it is not of the type detectable by Lucifer yellow transfer. Assays for electrical coupling or dye coupling using other tracers might reveal further functional regulation of Cx43 and the other connexins in cultured and flow-conditioned cells.

Our finding of low Cx43 flow regulation and participation in cellular coupling disagrees with earlier reports that in vitro Cx43 is upregulated by flow and, due to its abundant expression in culture, is the single most important endothelial connexin involved in functional gap junctional communication (10, 15, 16, 32, 37). Those studies were conducted in their majority with animal cells whose gap junctional connexin expression is significantly different from that of humans, dependent on species (44). Often, a single endothelial connexin (Cx43) was assessed, therefore leaving the potential participation of other vascular connexins unexplored. Furthermore, functional communication (dye and/or electrical coupling) studies were not able to differentiate the specific connexins involved in gap junction-mediated intercellular coupling. Our results corroborate in vivo studies that implicate Cx40 as the most prominent communicating connexin in human tissue followed by Cx37 (30).

In vivo abundant Cx43 expression has been associated with disturbed flow conditions at atherosclerotic lesion-prone regions of vessel constrictions and bifurcations, where no Cx37 and, in some cases, no Cx40 are found (21, 30). In vitro, Cx43 abundant expression by cultured human endothelial cells may be considered a culture artifact, nonessential and a marker of cellular dedifferentiation (10, 44). Cx43 uninvolved in functional communication resembling that of endothelial cells in vivo (21, 30) is not of the type detectable by Lucifer yellow transfer. Assays for electrical coupling or dye coupling using other tracers might reveal further functional regulation of Cx43 and the other connexins in cultured and flow-conditioned cells.

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endothelial cells in vitro. The low level of communication of these cells in culture, reported here for the first time, is unexpected when compared with their animal counterparts (2, 10, 16, 32, 39) and, moreover, reveals the dedifferentiation of an important cellular phenotype, cell communication relevant to other key cellular functions (23, 40). The fact that these cells regain communication in flow indicates that flow-conditioned cells may provide a more realistic model for studying human endothelial cell communication. In particular, flow-conditioned HAEC can be used to examine potential mechanisms in regulation of connexin expression and assembly into functional channels. Studies are currently ongoing in our laboratory to confirm a potential mechanism involving flow-mediated PKA signaling in connexin phosphorylation and nitric oxide-dependent expression (26, 46). The outcome of these experiments will add great depth to our understanding of the underlying mechanisms in regulation of endothelial gap junctional communication and the implication for relevant cellular functions and vascular tissue homeostasis.

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Flow Regulation of Cx40 and Cx37 Functional Channels

REFERENCES


