Connexin40 and connexin43 in mouse aortic endothelium: evidence for coordinated regulation

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Submitted 6 September 2005; accepted in final form 7 November 2005

Isakson, Brant E., David N. Damon, Kathleen H. Day, Yongbo Liao, and Brian R. Duling. Connexin40 and connexin43 in mouse aortic endothelium: evidence for coordinated regulation. Am J Physiol Heart Circ Physiol 290: H1199–H1205, 2006. First published November 11, 2005; doi:10.1152/ajpheart.00945.2005.—In the vessel wall, endothelial cells are metabolically and electrically coupled to each other and to the adjacent smooth muscle cells by gap junctions composed of connexins. Gap junctions may be formed from combinations of several different connexin proteins, and deletion of one connexin can lead to modification of the expression of another. To reveal a possible interaction between connexin40 (Cx40) and connexin43 (Cx43) in endothelium, we studied their distribution in vessels from C57Bl/6 and Cx40 knockout mice (Cx40−/−) using immunoblots and immunocytochemistry on aortic cross sections and en face whole mounts. En face preparations from C57Bl/6 mice revealed two distinct pools of Cx43, one pericellular and the other intracellular. Cx40 was largely restricted to the periphery of the cells, and in Cx40−/− mice it was, as expected, undetectable. In the Cx40−/− mice, total Cx43 protein was also modestly reduced (immunoblots), but there was a major redistribution of the protein within the cell. The pericellular component of Cx43 was rendered virtually undetectable, and the intracellular compartments were normal or even slightly elevated. Smooth muscle Cx43 was also reduced in the Cx40−/− animals. These findings indicate that the cellular distribution of Cx43 is dependent on the presence of Cx40, and in view of the profound effects on the pericellular pool of the Cx43, the findings suggest that interactions between Cx40 and Cx43 regulate communication between endothelial cells and perhaps between smooth muscle and endothelial cells as well.

METHODS

Animals and animal care. All procedures and protocols in this study were approved by the University of Virginia Animal Care and Use Committee. Male C57Bl/6 mice (Taconic Laboratories, Germantown, NY) or Cx40−/− mice (the kind gift of Drs. A. Simon, University of Arizona, and D. Paul, Harvard University) were used for these studies. Aortic and cardiac samples from Cx43−/− neonates were obtained by crossingbreeding Cx43−/− mice (Jackson Laboratories, Bar Harbor, ME) and harvesting tissues immediately after birth. Genomic DNA from tail clips confirmed the genotype of each animal studied.

RNA isolation and processing. RNA was isolated by using the Ambion RNequeous-4PCR kit and reverse transcribed by using Invitrogen Superscript RT-PCR reagents. For Cx43 PCR, we used the primer pair 5′-GACTGCGGATCTCCAAAATA and 3′-AAATCA-AACGGCTGGCCGTGG. For Cx40, we used 5′-CACCTGGGCTGGAAGAGAT and 3′-AGTGCCAGAGCCTCGATT. Platelet endothelial cell adhesion molecule (PECAM) and desmin were used as endothelial cell- and smooth muscle-specific markers, respectively, and were detected by using published primer sets (22, 24). Taq-only controls showed no contaminating DNA (data not shown).

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**Immunocytochemistry.** Immunocytochemistry was performed on unfixed frozen sections (31), fixed paraffin sections (19), and fixed en face whole mounts. Adult mice were anesthetized with pentobarbital sodium (50 mg/kg ip). The chest cavity was opened, and a needle was inserted into the left ventricle. The right ventricle was cut, and 5 ml of wash solution [MOPS-buffered physiological salt solution (PSS) containing 4 mM acetylcholine, 4 mM sodium nitroprusside, 5 U/ml heparin, and 1% fetal calf serum] were rapidly infused. Sixty milliliters of either 2% paraformaldehyde in MOPS-buffered PSS or Bouin’s fixative (11) in MOPS-buffered PSS were then perfused through the vasculature. For en face observation, the aorta was removed, the adventitial surface was cleaned of connective tissue, and the vessels were cut into segments 3–4 mm in length. Each segment was opened longitudinally and transferred to a glass slide (adventitial side down), and the segment edges were secured to the slide by using silicon rubber adhesive. Aortic segments were pretreated in MOPS-buffered PSS with 1% Triton X-100 and then exposed to primary antibody overnight at 4°C, after which they were washed and exposed to the secondary antibody for 1 h at room temperature. Pericellular boundaries were defined by costaining with vascular endothelial-cadherin and cortical actin. Aortic segments and sections were examined by using an Olympus Fluoview confocal microscope with a H1200 CX40 AND CX43 COREGULATION IN ENDOTHELIUM

**RESULTS**

**Intracellular Cx43 protein distribution in endothelium from C57Bl/6 and Cx40−/− mice.** En face immunocytochemistry was used to assess Cx43 protein expression in the presence and absence of Cx40. The C57Bl/6 aortas stained for Cx43 demonstrated extensive intracellular and pericellular staining (Fig. 1, A and B). Endothelium from the Cx40−/− mice showed a striking reduction in Cx43 pericellular stain while retaining substantial intracellular label (Fig. 1, C and D).

Cx43 and Cx40 protein distribution across the vessel wall. Frozen and paraffin-embedded cross sections were also examined to ascertain the distribution of the connexin proteins across the vessel wall in both smooth muscle and endothelium. Cx43 was present in both cell types, whereas Cx40 was detectable only in the endothelium (Fig. 2, A and B). The reduction of punctate staining of Cx43 in the endothelium of Cx40−/− animals was evident, but, unexpectedly, we observed that Cx43 punctates were also reduced in the smooth muscle of the Cx40−/− animals (Fig. 2C), even though there was no Cx40 detectable in the smooth muscle of the wild-type animals (Fig. 2, B and D). This qualitative observation was also noted in vascular endothelial cells Cx43−/− mice (19).

**Tests of connexin antibody specificity: Cx43−/− mice.** We made exhaustive tests to ensure that the apparent changes in Cx43 were not due to antibody cross-reactivity. We examined the Cx43 staining in aortic cross sections from Cx43−/− mice. The early postnatal lethality of this genotype necessitated the use of 1- to 6-h-old postnatal mice. In aortas of neonatal C57Bl/6 mice, Cx43 was found in both endothelium and vascular smooth muscle (Fig. 2E), whereas Cx40 was only found in endothelium (Fig. 2F). In neonatal aortas of Cx43−/− mice, no Cx43 staining was observed (Fig. 2G), whereas Cx40 was well expressed in endothelium (Fig. 2H).

**Tests of connexin antibody specificity: multiple antibodies.** We also made comparative measurements on a number of additional antibodies in the en face measurements. All Cx43 antibodies demonstrated extensive pericellular stain and varying degrees of intracellular and nuclear label (Fig. 3, A–D and F–I). Similar patterns of pericellular staining were seen with Bouin’s (Fig. 3, A–D) and paraformaldehyde (Fig. 3, F–I) fixation, although Bouin’s fixed tissue tended to show a more prominent pericellular distribution. In contrast to the Cx43 antibodies, Cx40 antibodies showed little evidence of cytoplasmic or nuclear staining (Fig. 3, E and J).

**Tests of connexin antibody specificity: peptide block of Cx43 staining.** As a further test of the specificity of residual Cx43 staining in endothelium, Cx43 antibodies were preincubated with a Cx43-GST protein. Preincubation with the GST protein blocked both the nuclear and the pericellular component of the Cx43 labels in both C57Bl/6 and Cx40−/− mouse aortic endothelium (Fig. 4). Taken together, these data lend strong evidence in support of the conclusion that the nonpericellular, residual punctata seen in en face Cx40−/− endothelium is Cx43 protein.
Measurement of Cx43 and Cx40 mRNA and protein in mouse aortic endothelium. To further analyze the alterations in protein expression in the Cx40−/− mice, we assayed the mRNA from endothelial cell isolates. Figure 5A shows mRNA from C57Bl/6 mice (lanes 1, 3, 5, and 7) and from Cx40−/− mice (lanes 2, 4, 6, and 8). Cx43 mRNA was readily detected in isolated endothelial cells from both C57Bl/6 (Fig. 5A, lane 1) and Cx40−/− mice (Fig. 5A, lane 2), as expected from previous work (31, 33). Cx40 mRNA was not found in the Cx40−/− mice (Fig. 5A, lanes 3 and 4). Endothelial cell mRNA from the mouse aorta was positive for PECAM and negative for desmin, demonstrating no smooth muscle cells in the isolation procedure (Fig. 5A, lanes 5–8).

The purity of the endothelial cells was further confirmed at the protein level by immunoblotting C57Bl/6 (Fig. 5, B–G, left lanes) and Cx40−/− (Fig. 5, B–G, right lanes) endothelial cells. Cells from both animals stained for claudin-5 (Fig. 5B) but not desmin (Fig. 5C), again indicating an absence of smooth muscle contamination. Cx43 protein was detected by immunoblot in the endothelium of both C57Bl/6 and Cx40−/− mice regardless of the Cx43 antibody used (Fig. 5, D and E). As expected, Cx40 protein was present in endothelium from C57Bl/6 but not Cx40−/− mice (Fig. 5F).

DISCUSSION

An altered phenotype from C57Bl/6 animals, including bradycardia and hypotension, is found in the endothelial cell-specific Cx43−/− animal (19), and this finding is difficult to reconcile with the reported absence of Cx43 protein in the mouse endothelium (15, 16, 30, 33). The absence of Cx43 in the presence of an altered phenotype from C57Bl/6 mice also raised the possibility of nonspecific effects of the conditional knockout resulting from the use of the cre-recombinase system or an alteration in the function of the floxed Cx43 gene.

We therefore examined the expression of Cx43 in the murine endothelium using multiple antibodies and a variety of approaches. Immunocytochemistry showed both pericellular and intracellular components to the Cx43 protein with each of the antibodies tested (Figs. 1–3), and we found that Cx40−/− mice have an altered distribution of the Cx43 protein. That is, in the aortic endothelium from the Cx40−/− mice, Cx43 appears to remain intracellular rather than being transported to the plasma membrane. The location and pattern of the labeled intracellular epitopes make it likely that the protein observed is resident in intracellular organelles. We have attempted to colocalize the intracellular Cx43 with endosomes or lysosomes using appropriate labels but to date have not been successful (data not shown). The simplest explanation for the data is that functional pericellular junctions (plagues?) in the endothelium require the presence of both Cx43 and Cx40. The possibility that a small and perhaps functional pool of Cx43 was still expressed at the pericellular boundary should also be noted.

The loss of pericellular stain for Cx43 might reflect either a true loss of protein or a failure of the antibody to detect the protein. The antibody might not have access to the Cx43 protein as a result of some change in the accessibility, e.g., as a result of altered tertiary structure. Alternatively, transport of Cx43 to the plasma membrane might require the presence of Cx40 (see below), and in the absence of an accompanying Cx40, Cx43 accumulates in regions such as the Golgi. Cell fraction studies will be necessary to answer questions such as these.

Surprisingly, in the Cx40−/− animals, not only is the distribution of Cx43 in the endothelium altered, but the amount of Cx43 in the smooth muscle seems to be qualitatively reduced (Fig. 2E). Because we find no Cx40 in the smooth muscle, the change in smooth muscle Cx43 cannot be due to the same sort of interaction hypothesized for the endothelium. Perhaps Cx40
deletion in the endothelium reduces endothelial Cx43, and this, in turn, causes a reduction in the smooth muscle Cx43 through some feedback between the endothelium and the smooth muscle. In an earlier report (19), we noted that selective deletion of Cx43 in the endothelium also causes a reduction in the smooth muscle Cx43 expression. It should be noted that this phenomenon has been described in other reports, although the Cx43 protein was upregulated in the smooth muscle cells instead of downregulated as we have observed (31). Regardless, this suggests that the expression of Cx43 in the two mural cell types may be coordinated by as yet unknown mechanisms.

Several lines of evidence have recently implicated the importance of Cx40 and Cx43 interactions. In vitro models of the myoendothelial junction demonstrated that Cx43 and Cx40, but not Cx37, play an integral role in the gap junctions formed between endothelium and vascular smooth muscle (14). In addition, double knockout mice in which endothelial cell-specific Cx43−/− and Cx40 were simultaneously deleted have similar elevated blood pressures compared with Cx40−/− mice (unpublished observation). Thus the effect of a double knock-
out of vascular endothelial cells Cx43−/−/Cx40−/− or a Cx40−/− would have similar results if no Cx40 or Cx43 is expressed at the pericellular boundary in either knockout. This suggests that deletion of Cx40 and the resultant reorganization of Cx43 could induce a hypertensive response. Interactions between Cx40 and Cx43 might occur during the oligomerization process or during transport of functional connexins into the membrane. Because Cx43 tends to oligomerize in the Golgi, the possibility exists for selective organization of homomeric Cx43 hemichannels or multiple combinations of heteromeric Cx40-Cx43 hemichannels (34). Understanding how this process of oligomerization occurs is important for understanding the interactions between the two connexins, their cellular distributions, and their capacity to be physiologically coupled.

Our work also corresponds well with key observations made by Simon and McWhorter (31) concerning dye transfer in Cx40−/− mice. In Cx40−/− endothelium, microinjection of neurobiotin revealed a significant reduction of dye transfer compared with C57Bl/6 endothelium (31). This reduction of dye transfer was attributed to the corresponding Cx37 protein downregulation in the Cx40−/− mice because it was noted that the Cx37 was still at the pericellular boundary but in much lower amounts (31). This demonstrates a change in connexin protein expression, which may be due to changes in intracellular signaling (for review, see Ref. 27). Similar to Cx37, our

**Fig. 4.** Cx43 glutathione S-transferase (GST) fusion peptide blocks Cx43 labeling in mouse endothelium. Cx43S staining in PFA-infused aorta from C57Bl/6 (A and B) or Cx40−/− mice (C and D). Preincubation of Cx43 antibody with Cx43-GST peptide eliminates all staining for Cx43, regardless of pericellular (A) or intracellular (A and C) distribution. Red is connexin, and bar in A is 20 μm and is representative for A–D.

**Fig. 5.** RT-PCR and immunoblots on murine aortic endothelial cells. Isolated endothelial cells from C57Bl/6 (lanes 1, 3, 5, and 7) and Cx40−/− (lanes 2, 4, 6, and 8) mice were subject to RT-PCR (A). Cx43 mRNA was readily detected in both C57Bl/6 and Cx40−/− animals (lanes 1 and 2). Cx40 mRNA was in C57Bl/6 endothelium but not in Cx40−/− mice (lanes 3 and 4). mRNA from endothelium of both types of animals was positive for platelet endothelial cell adhesion molecule (PECAM; lanes 5 and 6), whereas smooth muscle marker desmin was consistently absent (lanes 7 and 8). Immunoblots of endothelial cells isolated from C57Bl/6 (left lanes) or Cx40−/− (right lanes) mice (B–G). Immunoblots of isolated endothelium confirmed RT-PCR by demonstrating that endothelial cell phenotypic marker claudin-5 was present (B) but not desmin (C). With use of two different antibodies, Cx43 protein was apparent in both C57Bl/6 and Cx40−/− endothelial cells lysates (D–E). Cx40 protein was found in C57Bl/6 endothelium but was undetectable in lysates of Cx40−/− endothelial cells (F). β-Actin is shown as a comparison of loading levels of protein (G). Molecular weight marker in G is representative for D–F as well. Immunoblots were obtained from pooled endothelial cell isolates from 6 mice, and results were replicated 4 times.
results demonstrate that Cx43 protein is also downregulated, albeit away from the pericellular boundary. Together, it appears that all of the endothelial cell connexins are in some form expressed at lower levels or reorganized when Cx40 protein is eliminated. In this way, Cx40 may be acting as a “linchpin” for organization of the connexins into specific domains or hemichannel organizations. This is a key observation when evaluating the several physiological effects of Cx40 deletion already described (5, 10).

We find that Cx43 is well expressed in the mouse endothelium, and the reasons for prior reports that Cx43 is absent are unclear (15, 17, 31). Concerns of antibody cross-reactivity in this work are greatly reduced by several observations. First, four different antibodies from a variety of sources and directed toward different amino acid sequences and with different sensitivity to the phosphorylation state of the protein (6) showed qualitatively similar patterns of distribution (Fig. 3). The selectivity of the Cx43 antibodies is further supported by the lack of nonspecific label after application of the GST fusion peptide (Fig. 4). Moreover, in Cx43−/− embryos, the Cx43 signal was abolished (Fig. 2). Thus we conclude that the Cx43 protein is present in the mouse aortic endothelium. Simon and McWhorter (31) used different antibodies, and Fig. 3 highlights that there are differences between the affinity of the primary antibodies and the epitope. It is also conceivable that because of the interaction between Cx40 and Cx43 described herein, antibody access to the Cx43 epitope was hindered. Another possibility is the differences in species processing of antibodies used to detect specific antigens (1). However, Kruger et al. (15) investigated the activation of the Cx43 promoter using lac-Z as an indicator of connexin gene regulation and also failed to see evidence for Cx43 expression in the endothelium. It is, of course, possible that the lac-Z reporter had inadequate sensitivity to detect the activation of the promoter, but Kruger et al. (15) also failed to see Cx43 using techniques very similar to ours. An alternative and bothersome explanation is that the mouse strains used here and in the report by Kruger et al. (15) originate from quite different substrains of the C56Bl/6 mouse and that their Cx43 gene expression is in fact quite different from that in the strain that we used (32).

In summary, we have shown that Cx43 protein is present in the aortic endothelium and that its distribution is dependent on the presence of Cx40. The Cx43 protein, although still expressed in Cx40−/− endothelium, is selectively eliminated at the pericellular boundary, whereas the intracellular component is well maintained or enhanced. Taken together with the physiological alterations in Cx40−/− mice, we conclude that Cx40-Cx43 interactions in the endothelium play a vital role in determining vascular function.

ACKNOWLEDGMENTS

We thank Charles D. Little for insightful discussion on immunocytochemistry staining and protocol. We also thank Susan I. Ramos, Xavier F. Figueroa, and Ivan Rubio-Gayoso for valuable discussion. We are grateful to the University of Virginia Research Histology Core for expert tissue embedding and sectioning.

GRANTS

This work was supported by the National Heart, Lung, and Blood Institute Grants HL-55318 (to B. R. Duling) and HL-72864 (to B. R. Duling) as well as an American Heart Association Beginning Grant-in-Aid 0556319U (to B. E. Isakson) and a University of Virginia Cardiovascular Research Center Partners Fund Grant (to B. E. Isakson).

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