Connexin37 Is the Major Connexin Expressed in the Media of Caudal Artery

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Objective—To determine the connexins (Cxs) involved in intercellular coupling within vascular muscle, the present study has quantified mRNA and protein expression for Cx37, Cx40, Cx43, and Cx45 in the caudal artery (CA) and thoracic aorta (ThA) of the rat.

Methods and Results—Real-time polymerase chain reaction and immunohistochemistry identified Cx37 as the most abundantly expressed Cx in the CA, with fine punctate staining observed in the media. Conversely, mRNA for Cx43 was 40-fold greater in the ThA than in the CA, with punctate staining in the endothelium and media of the ThA but confined to the endothelium in the CA. Western blotting confirmed the differences in the relative amounts of Cx40 between the 2 vessels. For both arteries, Cx45 was expressed to a lesser degree in the media but not in the endothelium, whereas Cx40 was found only in the endothelium. Cx37, Cx40, and Cx43 were expressed in the endothelium of both vessels, although the density of Cx40 plaques was significantly greater in the CA.

Conclusions—The demonstration of Cx37 as the dominant Cx in the media of the CA highlights the potential heterogeneity in Cx involvement in vascular smooth muscle. (Arterioscler Thromb Vasc Biol. 2002;22:1427-1432.)

Key Words: connexin ■ endothelium ■ smooth muscle ■ thoracic aorta ■ caudal artery

Gap junctions are intercellular channels composed of membrane proteins known as connexins (Cxs), of which 4 (Cx37, Cx40, Cx43, and Cx45) have been identified in vascular tissue. In blood vessels, gap junctions are found connecting adjacent endothelial cells, connecting adjacent smooth muscle cells, and connecting endothelial and smooth muscle cells. Although 3 of the 4 vascular Cxs have been shown to be expressed by endothelial cells of most vessels, the identity of the Cxs connecting adjacent smooth muscle cells in arteries is less clear.

In large elastic arteries, such as the aorta, Cx43 is thought to be the major gap junctional protein expressed in the smooth muscle. More recently, some studies have shown expression of other Cxs in addition to Cx43 in the media of elastic arteries, although some of these differences can be attributed to heterogeneity among animal species. For example, Cx40 has been shown in the aorta of the cow and pig but not in the rat, whereas Cx37 has been reported in the aorta and pulmonary artery of the rat in some studies but not in others.

In muscular arteries, identification of the major Cx isoform has been more difficult. Cx43 has not been found in the media of a number of large muscular arteries, including the caudal, basilar, mesenteric, and coronary arteries, although it has been reported in pial and cremaster arterioles in rats and cheek pouch arterioles in hamsters. On the other hand, Cx40 appears to be a potential candidate in the media, having been identified in the coronary artery in a number of species, in the basilar artery, and in a number of different arteries in rats. Cx45 is also receiving some attention because of its identification in arterial smooth muscle in embryonic and adult mice. On the other hand, Cx37 is generally considered to be an endothelial Cx, although it has been described in the media of the larger coronary arteries of the rat and in the media of collateral vessels during coronary arteriogenesis in dogs.

Considering the absence of a clearly identifiable Cx in the media of muscular arteries and the potential for some confusion due to nonspecificity of antibodies, we chose to compare Cx expression at mRNA and protein levels in a large muscular artery with expression in an elastic artery. By using real-time polymerase chain reaction (PCR), we have been able to quantify mRNA expression in the 2 vessels and relate this to protein expression by using immunohistochemistry and Western blotting with the use of different sources of antibodies raised against different epitopes for which results might be contentious. Together, the mRNA and protein data are consistent with the predominant expression of Cx43 in the media of the thoracic aorta (ThA) but with the expression of Cx37 in the media of both arteries. Our results also demonstrate variation in the relative appearance of the phosphorylated and nonphosphorylated forms of Cx43 in different tissues.
Expression of mRNA for Vascular Cxs

Expression of Protein for Vascular Cxs

Methods

Results

Expression of mRNA for Vascular Cxs

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Immunohistochemistry

Results

Expression of mRNA for Vascular Cxs

Quantification of mRNA expression of the vascular Cxs with the use of real-time PCR was achieved by constructing standard curves from the plasmid clones of the 4 Cxs (see online Figure IA and IB, available at http://atvb.ahajournals.org, for Cx40). Expression of mRNA in the arterial samples was expressed as Cx copy number and was normalized to the number of copies of 18S RNA. Data showed that in the ThA, expression of mRNA for Cx37, Cx40, and Cx45 was significantly greater than expression of mRNA for Cx37, Cx40, and Cx45 ($P<0.05$, Figure 1). In the CA, expression of mRNA for Cx37 was significantly greater than expression of mRNA for Cx40, Cx43, and Cx45 ($P<0.05$, Figure 1). Expression of Cx37 mRNA was also significantly greater in the CA than in the ThA ($P<0.05$, Figure 1), whereas mRNA for Cx43 was significantly greater in the ThA than in the CA ($P<0.05$, Figure 1). Expression of mRNA for Cx37 in the CA was not significantly different from mRNA expression for Cx43 in the ThA. Expressions of mRNA for Cx40 and Cx45 were not significantly different between the 2 arteries (Figure 1).

Expression of Protein for Vascular Cxs

Immunohistochemistry

The distribution of Cx37 immunolabeling, typical for each of the 2 vessels, was the same regardless of whether the commercial Cx37 antibody or the affinity-purified Cx37/266 antibody was used. When tested on sections of rat lung, Cx37/266 showed abundant expression of Cx37 within the endothelium of blood vessels throughout the lung and within the smooth muscle layers of the bronchioles, consistent with studies of Nakamura et al.10 Staining was completely blocked by peptide (online Figure II, available at http://atvb.ahajournals.org). On the other hand, the commercial Cx40 antibody cross-reacted with Cx43, as evidenced by punctate staining in the media of the ThA and by the appearance of bands in Western blots of brain tissue, equivalent in size to those stained with antibodies against Cx43. Consequently, only results obtained with Cx40/254 are discussed. Staining with the 2 antibodies against Cx45 also revealed differences in sections of the ventricle but not in the 2 arteries. In the ventricle, the commercial anti-Cx45 stained cardiac myocytes throughout the myocardium, whereas Cx45/354 stained only the myocytes along the endocardial border, consistent with the restricted distribution found by Coppen et al.26 who used an antibody against the same sequence (see Figure 2E and 2F).

For each of the Cx antisera used in the present study, no staining at all was observed in the absence of the primary antibody or when the primary antibody was preincubated with the appropriate antigenic peptide.

Smooth Muscle Cells

Longitudinal and transverse sections of the rat ThA and CA were analyzed for expression of Cx37, Cx40, Cx43, and Cx45. No significant difference was found in the data for either orientation for any of the 4 Cxs in either artery. In the media of the CA, Cx37 was highly expressed (Figures 2A and 3A), whereas Cx37 expression in the ThA was very sparse ($P<0.05$, Figure 3A and online Figure IIIA, available at http://atvb.ahajournals.org). Expression of Cx40 was absent from the media of both arteries (Figures 2B and 3A and online Figure IIIB).

Cx43 was abundantly expressed in the media of the ThA (Figure 3A and online Figure IIIB) but was absent from the media of the CA (Figures 2C and 3A). Cx45 could be detected sparsely in the media of CA and ThA (Figures 2D and 3A and online Figure IIID).

Plaques of Cx37 and Cx45 in the media of the CA and Cx45 in the ThA were significantly smaller than Cx43.
plaques in the ThA (P<0.001; for CA, 0.08±0.003 μm [Cx37] and 0.06±0.001 μm [Cx45]; for ThA, 0.11±0.01 μm [Cx43] and 0.06±0.004 μm [Cx45]).

Endothelial Cells
En face views of the luminal surface showed punctate staining for Cx37, Cx40, and Cx43 along the periphery of endothelial cells in ThA and CA (online Figures IVA, IVB, and IVC, respectively [available at http://atvb.ahajournals.org], and Figure 4A, 4B, and 4C, respectively). Cx45 was not found in the endothelium of either artery (online Figure IVD and Figure 4D). The area, length, and width of endothelial cells did not differ between the CA and the ThA, although the perimeter of endothelial cells was significantly greater in the CA than in the ThA (P<0.05; online Figure V, available at http://atvb.ahajournals.org). Expression of Cx37 and Cx43 per square micrometer of luminal surface was similar between the ThA and CA, whereas expression of Cx40 was significantly less in the ThA than in the CA (P<0.05, Figure 3B). Because Cx expression in the endothelium was seen exclusively around the cell periphery (online Figure IV and Figure 4), the density of Cx plaques in the cell membrane was also calculated. The density of Cx40 plaques was significantly greater in the CA than in the ThA, whereas no such difference was seen for Cx37 and Cx43 (P<0.05). Plaque sizes for each Cx did not vary between endothelial cells in either artery. However, in the CA, Cx37 plaques between endothelial cells were significantly larger than plaques between smooth muscle cells (0.150±0.01 μm for endothelial cells and 0.075±0.003 μm for smooth muscle cells, P<0.0001), whereas in the ThA, plaques for Cx43 were greater in the endothelium than in the smooth muscle (0.143±0.01 μm for endothelial cells and 0.106±0.01 μm for smooth muscle cells, P<0.001).

Western Blotting
Rabbit Cx43 antibodies revealed the presence of multiple isoforms of Cx43 in extracts of brain, heart, lung, and ThA (Figure 5), with the higher molecular weight isoforms appearing to predominate in the heart and ThA (Figure 5, −control peptide). All isoforms were blocked by preincubation of the Cx43 antibody with immunogenic peptide (Figure 5, +control peptide). Cx43 was detectable only in extracts of CA when 10 μg of protein was loaded on the gel (online Figure VI, available at http://atvb.ahajournals.org) but not when 5 μg was used (Figure 5). Quantification of Cx43 by phospho-imaging revealed that compared with CA, ThA contained at least 9 times more Cx43 (data not shown). When samples of heart, ThA, and CA were treated with phosphatase, a shift in
mobility toward the lower molecular weight form was found (online Figure VI), confirming that the high molecular weight forms were phosphorylated Cx43, as previously demonstrated.27,28

The Cx40/254, Cx45/354, and commercial Cx45 antibodies all stained numerous bands in Western blots, although only a small number of bands disappeared when the antibody was preincubated with immunogenic peptide (online Figure VII, available at http://atvb.ahajournals.org). Thus, Cx40/254 antibody specifically recognized a band of 40 kDa from lung, CA, and ThA but not liver (online Figure VIIA, control peptide). In the lung, however, a band at 45 kDa also appeared to be reduced. The Cx45/354 antibody revealed the presence of a specific 45-kDa band in all tissues tested, although it was very weak in the arteries. A higher molecular weight band, which was blocked by peptide, was also seen in the brain (online Figure VIIIB, −/+peptide). On the other hand, the commercial Cx45 antibody labeled a 45-kDa band and also a lower molecular weight species in brain, heart, and CA (online Figure VIIIC, asterisk). Although the Cx37/266 antibody and the commercial Cx37 antibody worked well in immunohistochemistry, numerous bands were stained in Western blots, but none disappeared after peptide incubation.

**Discussion**

Significant differences have been found in the Cx makeup of the media, but not the endothelium, of an elastic and a muscular rat artery by using real-time PCR, immunohistochemistry, and Western blotting. In the CA, mRNA for Cx37 was the most prevalent of the 4 Cxs, and immunohistochemically demonstrable staining was found in the media of that artery, but it was not found to any great extent in the ThA. In the present study, which used high-resolution confocal microscopy, a single fluorescent spot was defined as a gap junctional plaque, as validated previously. On the other hand, mRNA for Cx43 was the most prevalent Cx in the ThA and greatly exceeded the expression of Cx43 in the CA. In similar fashion, the specific activity of Cx43 protein on Western blots was significantly greater in the aorta than in the CA, in agreement with the extensive punctate staining in the aortic media and an absence of staining in the CA, as described previously. In the endothelium, protein for Cx37, Cx40, and Cx43 was detected in both vessels, as was Cx45 in the media of the 2 vessels. These data strongly suggest that the predominant Cx connecting smooth muscle cells in the CA is Cx37, in contrast to the aorta, where the predominant Cx is confirmed to be Cx43, with Cx45 playing a minor role in both arteries.

Although the mRNA extracts of both arteries included smooth muscle cells and endothelial cells, the predominant cell type was the smooth muscle cell, inasmuch as our ultrastructural studies have demonstrated that there are ≥7 layers of smooth muscle cells surrounding the single layer of endothelial cells in the 2 vessels (S. Sandow, unpublished data, 2002). Thus, when protein expression for each of the Cx subtypes in the media of the 2 arteries was compared with the mRNA expression in the vessels, a good correlation was found for all 4 Cxs in both arteries. In contrast, in cultured
smooth muscle cells from preglomerular arterioles of the rat, only Cx40 protein was detected in spite of mRNA expression for Cx37, Cx40, and Cx43.18 Although Cx40 protein was also found in sections of preglomerular arterioles in vivo, no mRNA analyses of this Cx or of other Cxs were performed.18 In preliminary experiments for the present study, attempts were made to rub off the endothelium to attribute mRNA for specific Cx isoforms to specific cellular layers. Unfortunately, these experiments were not entirely successful, inasmuch as real-time PCR showed that expression of mRNA for the endothelial cell marker von Willebrand factor could still be detected in these “endothelium-denuded” preparations.

Although Cx37 was extensively expressed in the media of the CA, it was effectively absent in the media of the ThA. These results are in agreement with the study of van Kempen and Jongsmna,11 who failed to find any Cx37 labeling in the aortas of several species, but in contrast to the study of Nakamura et al,10 who described the presence of Cx37 in the smooth muscle of the rat aorta and pulmonary artery. However, the authors did not define which region of the aorta was used, and because expression of other Cxs has been shown to vary along the length of the aorta,18,20 it may be difficult to directly compare the results. Expression of mRNA for Cx37 was 10-fold greater in the CA than in the ThA, in line with the observed staining within the muscle layers. In the same species, Cx37 has also been identified in the media of large coronary arteries,11 perhaps suggesting a more widespread role for Cx37 in cell coupling in the media of large muscular arteries in the rat.

In the present study, Cx40 was not detected in the media of either the ThA or CAs. These results are in contrast to several previous studies that have described Cx40 in the media of blood vessels from several species, including preglomerular and pial arterioles of the rat,16,18 hamster cremaster muscle arterioles,16 and coronary arteries from the cow, pig, and rat.11 Taken together, these results may suggest a greater role for Cx40 in smaller vessels, supporting the idea that heterogeneity exists in the expression of Cxs within different parts of the vascular tree.

In the ThA, Cx45 was relatively sparsely expressed in contrast to the dense expression of Cx43. A reciprocal relationship between these 2 Cxs was found throughout the aortic vessels to the iliac artery.20 In the CA, double labeling with antibodies against smooth muscle myosin showed Cx45 between smooth muscle cells, and with the use of real-time PCR, expression was similar to that in the ThA. The greater expression of Cx37 than of Cx45 in the CA suggests that Cx37 may share a similar inverse relation to Cx45 in the media of the CA. Alternatively, expression of the 2 Cxs may have implications for radial versus longitudinal coupling of smooth muscle cells. Recent dye-coupling studies in the CA from our laboratory (N. Bramich, unpublished data, 2001) have shown selective spread of Lucifer yellow dye between smooth muscle cells in the radial, but not the longitudinal, direction. However, in the present study, in the media of both arteries, protein expression of Cx37 and Cx45 did not differ between longitudinal and transverse orientations.

As previously demonstrated, protein for Cx37, Cx40, and Cx43 was expressed in the endothelium of the ThA and CA,15 whereas the expression of Cx45 was not detected in the endothelium of either artery.2,20 Staining was essentially confined to the intercellular borders. The expression of Cx40 was significantly greater in the endothelium of the CA than in the ThA, whereas the expression of Cx37 and Cx43 was not significantly different between the 2 arteries. Morphological measurements demonstrated some differences in the shape of endothelial cells between the 2 arteries, resulting in an increase in cell perimeter in the CA relative to that in the ThA. In spite of this increase in cell perimeter, the density of Cx40 plaques was also significantly greater in the CA than in the ThA.

The validity of protein data ultimately depends on the specificity of the antibodies used. The commercial Cx43 antibody stained a number of molecular weight bands that were confirmed in the present study to represent unphosphorylated and phosphorylated Cx43.23 Using a similar antibody, Hossain et al27 found no differences in immunohistochemical staining of brain tissues in which different relative amounts of these phosphorylated forms existed. For each of Cx37, Cx40, and Cx45, 2 antibodies directed against well-separated epitopes were used. Both Cx37 antibodies yielded similar immunohistochemical results, specific for the 2 arteries. Furthermore, in the lung, there was extensive Cx37 staining of blood vessels and bronchioles as previously described,10 confirming specificity. A similar antibody to Cx37/266 has been previously characterized with the use of Western blotting of Cx-transfected cells.9,25 The Cx40/254 antibody detected a 40-kDa protein in Western blots of tissue samples (present study), and a similar antibody demonstrated specificity in Cx-transfected cells.9 However, the commercial antibody to Cx40 appeared to cross-react with Cx43, and use was discontinued. The Cx45/354 antibody produced restricted labeling of the ventricular endocardium consistent with that previously demonstrated by Coppen et al26 for an antibody raised against the same epitope and fully characterized in transfected cells.5 However, the commercial Cx45 antibody cross-reacted with Cx43, in a manner similar to that described by Coppen et al,26 for a similarly directed commercial Cx45 antibody. Interestingly, the commercial Cx45 antibody did not stain the muscle of the aorta or the endothelium of either vessel, which are sites of extensive Cx43 expression. Coppen et al26 have elegantly identified the cross-reacting peptide as residues 283 to 286 of Cx43. We found that this site lies in an area of the carboxy terminus rich in predicted phosphorylation sites, immediately adjacent to a serine and containing a tyrosine. Thus, we suggest that tissue-specific phosphorylation of Cx43 occurs in the vessels but not in the heart, thus reducing accessibility of this antibody in the former but not in the latter.

Tissue-specific distribution of the unphosphorylated and phosphorylated forms of Cx43 has been previously reported.28 In the present study, the higher molecular weight phosphorylated forms predominated in the heart and ThA, whereas the lower molecular weight forms were more prevalent in the brain and CA. Hossain et al27 demonstrated that the predominance of the lower molecular weight forms in the brain was due to rapid dephosphorylation of Cx43. Because Cx43 in the CA is restricted to the endothelium, whereas
Cx43 in the ThA is predominantly in the smooth muscle, differences in specific kinases or phosphatases may exist between these 2 tissues.

The present study has demonstrated that Cx37 is the major Cx expressed in the media of the CA, whereas this role is played by Cx43 in the ThA. Reduced expression of Cx45 suggests that it plays a minor role in the media of both arteries, whereas Cx40 is not expressed in the muscle of either vessel. In contrast, 3 Cxs are expressed in the endothelium of both vessels, and of these, Cx40 is more prevalent in the CA than in the ThA.

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References

Connexin 37 is the major connexin expressed in the media of the caudal artery
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CONNEXIN 37 IS THE MAJOR CONNEXIN EXPRESSED IN THE MEDIA OF THE CAUDAL ARTERY

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ONLINE DATA SUPPLEMENT
MATERIALS AND METHODS

All experiments were conducted in line with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, under a protocol approved by the Animal Experimentation Ethics committee of the Australian National University.

RNA extraction and cDNA synthesis

Messenger RNA was extracted from intact thoracic aorta and caudal arteries dissected from 4-5 week old male Wistar rats, killed humanely with an overdose of ether anaesthetic. Vessels were removed into cold phosphate buffered saline (PBS), cleaned of adherent fat and stored in RNAlater (Ambion) before being homogenized in cold RNAzol B (Tel-test Inc.) for RNA extraction according to the manufacturer’s instructions. Four separate RNA extractions were prepared for each arterial type.

RNA was reverse transcribed (42°C for 1h, 50°C for 1h, 90°C for 10 min) in the presence of oligo dT primers (100 ng/µl, Stratagene) and reverse transcriptase (200 U/µl, GIBCO BRL). For each sample, a separate reaction was performed without the presence of reverse transcriptase enzyme to control for genomic DNA contamination.
Real time PCR

Quantitative real time PCR of cDNA samples obtained from thoracic aorta and caudal arteries was performed using the Applied Biosystems ABI Prism 7700 sequence detection system and SYBR green core reagents kit (Applied Biosystems). Reactions were performed in duplicate in a total reaction volume of 25 µl, each containing 50 ng of cDNA sample or 3 fold dilutions of plasmid standards. All samples were diluted in water containing tRNA (Sigma, final concentration of 1 ng / reaction). Reactions contained 1X SYBR green PCR buffer containing Passive Reference 1, 3 mM MgCl2, 1.5 mM dNTP mix and 0.625 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Primers specific for Cxs 37, 40 and 43 were designed from published rat sequences (see Supplementary Table 1). Since the rat sequence for Cx45 was not known, forward (5’-ACAAGAAGGCAGCTCGGAGCAA-3’) and reverse (5’-CAAGGAAGTCTGCTGCACACATA-3’) primers were designed to be 100% homologous with published human, mouse and wolf sequences. These primers amplified a product of 321 bp from rat caudal artery (annealing at 63°C for 10 sec, extension at 72°C for 35 sec), and this product was purified and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The rat Cx45 sequence obtained was aligned with the published human, mouse and wolf sequences and found to be 98% homologous to the corresponding mouse sequence and 93% homologous to the consensus sequence for the four species. New primers were designed specifically to the rat Cx45 sequence (see Supplementary Table 1), and these were used in subsequent real time PCR to amplify a product of 132 bp.
All primers were used at a concentration of 800 nM per reaction. Products were amplified using the following conditions, 13 min at 95°C, to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15 sec at 95°C, primer annealing for 15 sec at temperatures shown in Table 2, and primer extension at 72°C for the times shown in Supplementary Table 2. Each experiment included control reactions containing no enzyme or no cDNA, and RNA samples that had not been reverse transcribed, to test for amplification of genomic DNA. Increases in PCR product present in each sample were measured in the real time PCR system as increases in SYBR green fluorescence. The threshold cycle (Ct) value calculated by the sequence detection software is defined as the cycle number when fluorescence was first detected above background. PCR products for each primer set were run on 2% agarose gels to confirm the presence of a single band.

Quantification of samples was achieved after constructing standard curves using plasmid preparations containing each of the cloned PCR products. Standard curves were constructed in duplicate for each Cx by three-fold serial dilutions of the plasmid cDNA from $10^5$ copies. The logarithm of the input plasmid cDNA concentration, as expressed in copy number, was plotted versus the mean Ct value obtained from the duplicate samples. In order to normalise samples for variations in RNA extraction, real-time PCR of tissue samples was performed using primers directed against a short segment of the 5’ region of 18S ribosomal RNA (see Supplementary Table 1 and 2 for primer sequences and thermocycling conditions respectively) and standard curves were constructed using plasmid preparations containing the cloned PCR product. The number of copies of each Cx was then expressed relative to $10^8$ copies of 18S ribosomal RNA.
**Immunohistochemistry**

The thoracic aorta and caudal arteries from 4-5 week old male Wistar rats were prepared for immunohistochemical examination of the smooth muscle and the endothelium as tissue sections or as whole mount preparations respectively. To obtain tissue for cryosectioning, animals were anaesthetised with ether and killed by cervical dislocation. The thoracic aorta, caudal arteries, ventricles and lungs were removed and impregnated with 30% sucrose in phosphate buffered saline (PBS) before freezing in Cryo-M-Bed (Bright Instrument Company Ltd., England). 10 µm thick cryosections were cut transversely or longitudinally for each vessel and mounted on slides coated with 2% gelatin. To obtain tissue for en face examination of the endothelium, animals were anesthetized by intraperitoneal injection of ketamine / rompun (44 and 8 mg/kg respectively) and perfused at a pressure of 60 mmHg, with saline (0.9% NaCl) containing 0.1% NaNO₃, 0.1% bovine serum albumin and 5 units/ml heparin at 25°C, and then with 2% paraformaldehyde in 0.1 mol/L sodium phosphate buffer. Arteries were removed into PBS, cut into 5 mm long segments, cut open longitudinally and pinned flat on Sylgard (Dow Corning, USA).

Cryosections and whole-mount tissues were pre-incubated for 30 minutes in a blocking solution of 2% bovine serum albumin (BSA), 0.2% Triton-X in PBS, followed by incubation in either Cx37, Cx40, Cx43 or Cx45, diluted in blocking buffer. Cryosections were incubated in primary antibody solution (1:250 for Cx37, 40 and 43, 1:500 for Cx45) for 1 hour at room temperature, while whole-mount tissues were incubated in primary antibody solution (1:100) for 2 hours at 37°C. After washing in PBS, tissues were incubated for 1 hour at room temperature with Cy3-conjugated anti rabbit or anti-goat immunoglobulins (Jackson Immunoresearch Laboratories Inc, PA, 1:100) in 0.01% Triton-X in PBS. Some tissue samples were
subsequently incubated for 1 hour at room temperature with rabbit anti-human von Willebrand factor polyclonal antiserum (anti-vWF, 1:300; Dako, Denmark) to specifically detect endothelial cells. Other samples were labelled with rabbit anti-chicken gizzard smooth muscle myosin (kindly supplied by U. Groschel-Stewart, 1:100), at room temperature for 1 hour, to specifically label smooth muscle cells. After washing in PBS, tissues were incubated in swine anti-rabbit FITC (Dako, Denmark, 1:40), for 1 hour at room temperature, washed and mounted in buffered glycerol.

To test the specificity of each antibody, tissues were incubated either without primary antibody or with primary antibody that had previously been pre-incubated for 1 hour at room temperature with 10-fold excess by weight of the peptide against which the antibody was raised.

**Confocal Microscopy and Morphometric Analysis**

All immunolabeled tissues were viewed using a Leica confocal laser scanning microscope (TCS 4D, Leica Instruments, Austria), equipped with an argon/krypton laser and fitted with the appropriate filters for the detection of Cy3 and FITC fluorescence. Optical sections of smooth muscle and endothelial cell layers were simultaneously obtained for both Cy3 (Cx staining) and FITC (anti-vWF staining or α-smooth muscle myosin) labels. For tissue sections, optical sections were collected at 1 µm intervals throughout the section thickness, while for whole mount tissue, optical sections were collected at 1 µm intervals throughout the endothelial cell layer as determined by the extent of the anti-vWF labeling. Care was taken to maintain similar gain settings for comparisons of antibody staining in the two vessels. Series of images were recombined to create a single image incorporating all smooth muscle
or endothelial cell labeling. For each sample, images from 3 different fields were obtained for analysis and samples taken from 4 different animals.

**Western Blotting**

Brain, heart, liver, lung, thoracic aorta and caudal arteries were removed from 5-6 week old Wistar rats and snap frozen in liquid nitrogen. Tissues were ground under liquid nitrogen in a mortar and pestle and resuspended in 1ml of lysis buffer (1 mM NaHCO₃ pH 7.05, 10 mM EDTA, 10 mM Iodoacetamide, 10 mM tetra-sodium pyrophosphate, 1 mM PMSF and 1 μg/ml each of antipain, aprotinin, pepstatin-A, chymostatin and leupeptin). Tissues were further disrupted by grinding in a polytron blender. Unbroken cells and large debris were removed by centrifugation at 1000 g for 5 minutes at 4°C, the supernatant was then removed and centrifuged at 3000 g for 5 minutes. The pellet was discarded and the supernatant centrifuged at 20000 g for 15 minutes at 4°C. The supernatant was discarded and the membrane-enriched pellet was resuspended in lysis buffer. Protein concentration was measured using the Bio-Rad protein assay kit.

Membrane-bound connexins were subsequently solubilized by incubation in 2x SDS sample buffer (5% SDS, 125 mM Tris-Cl (pH 6.8), 20% glycerol, 2 mM β-mercaptoethanol, 0.1% (w/v) bromophenol blue) for 60 minutes at 37°C. Aliquots containing 5 μg of protein were separated by SDS-PAGE on 12% polyacrylamide gels, using Rainbow molecular weight markers (Amersham), and blotted onto Immobilon-P membranes (Millipore). Blots were probed with sheep antibodies against Cxs 37 (1:1000, Cx37/266), 40 (1:1000, Cx40/254) and 45 (1:500, Cx45/354), rabbit anti-Cx43 (1:1000) and the commercial rabbit anti-Cx37 (1:300, 1:1000) and anti-Cx45 (1:500). Western blots were developed using horse radish
peroxidase-conjugated donkey anti-sheep IgG (Jackson research laboratories, 1:4000) or goat anti-rabbit IgG (Sigma, 1:10000), and ECL chemiluminescence reagents (Amersham) according to the manufacturer’s instructions.

Identification of Cx43 isoforms was performed in samples of thoracic aorta, caudal artery and heart tissue, lysed in RIPA buffer (150mM NaCl, 1%NP40, 0.25% deoxycholic acid, 0.1% SDS, 1mM EDTA containing 1mM PMSF and 20µg/ml each of leupeptin, aprotinin, pepstatin, antipain and chymostatin according to the method of Nagy et al.1 The phosphatase inhibitors NaF (10mM) and sodium orthovanadate (10mM) were added to several samples of each tissue in order to compare the level of phosphorylation between samples. For dephosphorylation, samples were treated with calf intestinal alkaline phosphatase (CIP) (Boehringer) at a concentration of 1U/µg tissue. Samples containing CIP were incubated at 37°C for 30 minutes prior to solubilisation of all proteins in 5% SDS sample buffer. Samples (10 µg protein) were separated on an 8% PAGE gel and blotted onto Immobilon-P membranes (Millipore). Protein was detected using rabbit anti-Cx43 antibodies (Zymed) at a concentration of 1:1000. Blots were developed using an Amersham ECL kit as describe above.

**Statistical analysis**

Results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was tested using one way analysis of variance (ANOVA) followed by student t-tests with Bonferroni correction for multiple groups. A $P$ value of <0.05 was taken as significant.

**References**

**Supplementary Figure I.** Amplification plot of serially diluted Cx40 plasmid cDNA, showing duplicate samples (A). Plasmid standard curve constructed by plotting input plasmid cDNA copy number versus mean Ct value obtained from the duplicate samples (B).

**Supplementary Figure II.** Connexin 37 expression in the Wistar rat lung. Cx37 labeling is seen in the endothelium of blood vessels (bv) within the lung and in smooth muscle cells of the bronchiole (br) (A). Cx37 labeling is abolished when the antibody is incubated with antigenic peptide prior to incubating with tissue (B).

**Supplementary Figure III.** Cx expression in smooth muscle cells (sm) of the rat thoracic aorta. Transverse sections of the thoracic aorta were incubated with antibodies against Cxs 37/266 (A), 40/254 (B), 43 (C) and 45/354 (D). Cx43 (C) expression was detected extensively in the smooth muscle cells layers, while Cx45 was only detected sparsely in the smooth muscle. Expression of Cxs 37 (A) and 40 (B) were not detected in smooth muscle cells. Calibration bar represents 10 µm.

**Supplementary Figure IV.** *En face* view of Cx expression in endothelial cells of rat thoracic aorta. Cx 37 (A), 40 (B) and 43 (C) plaques can be seen outlining the perimeter of endothelial cells, while Cx45 (D) is not detected. Calibration bar represent 20 µm.
Supplementary Figure V. Morphology of endothelial cells in rat thoracic aorta (ThA) and caudal arteries (CA). Values are mean ± SEM. *P<0.05, significantly different from the ThA. n=4 animals.

Supplementary Figure VI. Tissue extracts (10 µg) from rat heart, CA and ThA were run on 8% SDS PAGE gels and probed with Cx43 antibodies. Extracts were prepared in buffer either containing (first panel) or not containing (second panel) the phosphatase inhibitors sodium fluoride and sodium orthovanadate (NaF/PhI) or were incubated with alkaline phosphatase (AlkPhos, third panel). Higher molecular weight forms of Cx43 were removed by treatment of all three tissues with alkaline phosphatase. Prot Inh, protease inhibitor.

Supplementary Figure VII. Western blotting of tissue extracts from rat brain, heart, lung, liver, caudal artery (CA) and thoracic aorta (ThA) using Cx40/254 (A), Cx45/354 (B) and commercial Cx45 (C) antibodies. Arrows show the position of the expected Cx bands. The left panels represent incubation with Cx antibody whereas right panels represent pre-incubation of the antibody with immunogenic peptide. Lower molecular weight proteins that cross-react with the commercial Cx45 antibody are marked (*).
**Supplementary Table 1.** Oligonucleotide primers used in Real time PCR reactions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin 37</td>
<td>AGCTCTGCATCCAAGAAGCAGTA</td>
<td>AGTTGTCTCTCAAGTGCCTTTGA</td>
<td>133</td>
</tr>
<tr>
<td>Connexin 40</td>
<td>GGAAAGAGGTGAACGGGAAGATT</td>
<td>CACAGCCATCATAAGACAAATGAA</td>
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<tr>
<td>Connexin 43</td>
<td>GAGATGCACCTGAAGCAGATTGAA</td>
<td>GATGTTCAAAGCGAGACACCAA</td>
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</tr>
<tr>
<td>Connexin 45</td>
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<td>CAAGGAAGTCTGCTGCACACATA</td>
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</tr>
<tr>
<td>18S</td>
<td>CCAGTAGCATATGCTTTGCTCAA</td>
<td>CGACCAAACCAACCATAACTGATT</td>
<td>112</td>
</tr>
</tbody>
</table>

**Supplementary Table 2.** Real time PCR thermocycling conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annealing temperature</th>
<th>Extension time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin 37</td>
<td>60°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>Connexin 40</td>
<td>60°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>Connexin 43</td>
<td>65°C</td>
<td>85 sec</td>
</tr>
<tr>
<td>Connexin 45</td>
<td>63°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>18S</td>
<td>61°C</td>
<td>60 sec</td>
</tr>
</tbody>
</table>
**Supplementary Table 2.** Real time PCR thermocycling conditions.

<table>
<thead>
<tr>
<th>Connexin</th>
<th>Annealing temperature</th>
<th>Extension time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>60°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>40</td>
<td>60°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>43</td>
<td>65°C</td>
<td>85 sec</td>
</tr>
<tr>
<td>45</td>
<td>63°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>18S</td>
<td>61°C</td>
<td>60 sec</td>
</tr>
</tbody>
</table>
Supplementary Figure V

Length (μm)

Width (μm)

Area (μm²)

Perimeter (μm)

ThA  CA  ThA  CA

*
**Supplementary Figure VI**

| Prot Inh | + | + | + | + | + | + | + | + |
| NaF/ Phl | - | - | - | - | - | - | - | - |
| Alk Phos | - | - | - | - | + | + | + | + |

<table>
<thead>
<tr>
<th>Heart</th>
<th>CA</th>
<th>ThA</th>
<th>Heart</th>
<th>CA</th>
<th>ThA</th>
<th>Heart</th>
<th>CA</th>
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<td></td>
</tr>
</tbody>
</table>

* Cx43
**
Supplementary Figure VII

A

- control peptide
+ control peptide

B

- control peptide
+ control peptide

C

- control peptide
+ control peptide