Polymorphism in Endothelial Connexin40 Enhances Sensitivity to Intraluminal Pressure and Increases Arterial Stiffness


Objective—To determine whether impairment of endothelial connexin40 (Cx40), an effect that can occur in hypertension and aging, contributes to the arterial dysfunction and stiffening in these conditions.

Approach and Results—A new transgenic mouse strain, expressing a mutant Cx40, (Cx40T202S), specifically in the vascular endothelium, has been developed and characterized. This mutation produces nonfunctional hemichannels, whereas gap junctions containing the mutant are electrically, but not chemically, patent. Mesenteric resistance arteries from Cx40T202S mice showed increased sensitivity of the myogenic response to intraluminal pressure in vitro, compared with wild-type mice, whereas transgenic mice overexpressing native Cx40 (Cx40Tg) showed reduced sensitivity. In control and Cx40Tg mice, the sensitivity to pressure of myogenic constriction was modulated by both NO and endothelium-derived hyperpolarization; however, the endothelium-derived hyperpolarization component was absent in Cx40T202S arteries. Analysis of passive mechanical properties revealed that arterial stiffness was enhanced in vessels from Cx40T202S mice, but not in wild-type or Cx40Tg mice.

Conclusions—Introduction of a mutant form of Cx40 in the endogenous endothelial Cx40 population prevents endothelium-derived hyperpolarization activation during myogenic constriction, enhancing sensitivity to intraluminal pressure and increasing arterial stiffness. We conclude that genetic polymorphisms in endothelial Cx40 can contribute to the pathogenesis of arterial disease. (Arterioscler Thromb Vasc Biol. 2013;33:962-970.)

Key Words: arterial dysfunction ■ connexin40 ■ endothelium ■ myogenic response ■ stiffness

Synchronization of cellular activity through gap junctions, and their constituent connexin (Cx) proteins, is essential for cardiovascular function, both at the level of the heart and the vasculature.1,2 During aging and cardiovascular disease, significant changes to Cx expression occur in the vasculature, and these alterations have been linked to the functional deficits found in these conditions.3,13 Expression of Cx37 and 40 is reduced in the endothelium of both muscular and conduit arteries during hypertension,4–7 along with gap junctional coupling in the media of muscular arteries8,14; whereas changes in Cx43 expression in conduit arteries seem to vary among different hypertensive models.9,10 Between the 2 vascular cell layers, the incidence of myoendothelial gap junctions is also altered during hypertension, correlating with changes in vasodilation resulting from endothelium-derived hyperpolarization (EDH).4,9,11,12 Similar changes in gap junction expression also occur during aging; endothelial Cx expression and myoendothelial gap junctions decrease11,13,14 in concert with the functional importance of EDH.15 What is not clear, however, is whether impairment of arterial gap junctions in ageing and hypertension is causal to, or a consequence of, the pathogenesis of arterial dysfunction.

Early efforts to attribute specific cardiovascular functions to different Cx subtypes involved creation of global knockout (KO) mouse strains. However, pinpointing the precise function of these proteins has been difficult as a result of embryonic lethality in the case of the Cx43KO and Cx45KO mice, and lack of cardiovascular phenotype in the case of the Cx37KO mice.16–18 In contrast, Cx40KO mice are profoundly hypertensive but display dysfunction of multiple cardiovascular organs, including the heart, kidney, and vasculature.19–22 The recent creation of mice with a renin-specific deletion of Cx40 has enabled the hypertensive phenotype to be attributed to ectopic localization of renin secreting cells and uncontrolled...
renin secretion. In support of this conclusion, mice with an endothelial-specific deletion of Cx40 are normotensive. Nevertheless, endothelial deletion of Cx40 does impair agonist-induced conducted vasodilation, a process which links tissue blood flow to metabolic activity, although effects on other vascular responses, such as the myogenic response, have not been tested. However, deletion of Cx40 in the global and promoter-specific mice is accompanied by coordinate downregulation of Cx37 in the endothelium, making attribution of vascular defects specifically to Cx40 difficult.

We have therefore developed a new strategy to investigate the role of endothelial Cx40 in vascular function. Rather than deleting Cx40, we have created a transgenic mouse strain that expresses a mutant form of Cx40, specifically in the endothelium, in addition to the endogenously expressed Cx40. The single-point site targeted for mutation lies in the center of a short 6 amino acid sequence previously shown to be critical for gap junction function. This strategy was chosen because it would allow us to also test the potential of a single-point Cx40 mutation, as it occurs in the human population, to alter vascular function. We hypothesized that Cx37 expression would not be altered because Cx40 would not be absent from the cell membrane. Using this model, we aimed to determine whether impairment of endothelial Cx40 affects vascular function, by studying the myogenic response and arterial stiffening, vascular characteristics which are altered in aging and hypertension.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Mutation at Position 202 Impairs Electric Conduction Through Hemichannels but Not Gap Junctions
The ability of the mutated form of Cx40, Cx40T202S, to form functional hemichannels and gap junctions was tested in isolated and paired Xenopus oocytes, respectively.

Isolated oocytes were divided into 2 groups based on the amplitude of their endogenous divalent-free Cx38 hemichannel currents. In oocytes with small endogenous currents, expression of wild-type Cx40 (wtCx40) produced a significant increase in hemichannel current (Figure 1A; P<0.05), whereas the Cx40T202S mutant induced no increase in hemichannel currents, whether injected alone or with wtCx40. In oocytes with large endogenous currents, wtCx40 had no further effect (Figure 1B; P>0.05). In contrast, expression of the Cx40T202S mutant significantly reduced the endogenous Cx38 current (P<0.05), but this could be restored by injection with wtCx40 (Figure 1B). Thus, the Cx40T202S mutant does not form functional hemichannels and shows a dominant-negative inhibition of the Xenopus orthologue of Cx37, but not of wtCx40 hemichannels.

Paired oocytes, in which endogenous Cx currents were suppressed with antisense oligonucleotides against Cx38, were electrically coupled to a similar extent after injection of both cells with either wtCx40 or Cx40T202S (Figure 1C). Similar electric coupling was obtained when oocytes expressing Cx40T202S were paired with those expressing wtCx40, or when oocytes expressing wtCx40 were paired with oocytes expressing both wtCx40 and Cx40T202S. Electrical coupling was also observed when Cx40T202S, with or without coexpression of wtCx37, was paired with wtCx37 (Figure 1D). These results surprisingly show that gap junction formation, as measured by electric coupling, is normal for the Cx40T202S mutant in homotypic, heterotypic (with wtCx40 or wtCx37), and even heteromeric configurations.

Mutation at Position 202 Impairs Chemical Conduction Through Gap Junctions
To examine the impact of the T202S mutation on junctional coupling in endothelial cells, we expressed wtCx40 or Cx40T202S in mouse coronary endothelial cells (MCECs) and used the ester (calcine-AM) transfer (or parachute) assay. Both Cx40 transgenes expressed well, whereas there

![Image](http://atvb.ahajournals.org/)

**Figure 1.** Cx40T202S mutant forms electrically impaired hemichannels, and chemically, but not electrically, impaired gap junctions. A, Expression of wtCx40 (Cx40), with or without Cx40T202S, in low Cx38 expressing oocytes, induces peak, divalent-free current (I_{DVF}), whereas expression of Cx40T202S alone has no effect. B, Expression of Cx40T202S in high Cx38 expressing oocytes reduces peak current. Coexpression with Cx40 reverses this effect. C, Normalized conductance between oocyte pairs demonstrates functional gap junctions formed by Cx40 or Cx40T202S in heterotypic and heteromeric combinations. D, Electrical conductance is normal for heterotypic and heteromeric gap junctions of Cx40T202S with Cx37. E, Western blot of endothelial cell line homogenate demonstrating expression of Cx40 transgenes. F, Percentage change in dye transfer from endothelial cell lines transfected with wtCx40 or Cx40T202S constructs. *P<0.05 compared with native/empty vector, †P<0.05 compared with wtCx40 injection. n= no. of oocytes (A–D), experiments (F).
was no expression of endogenous Cx40 protein (Figure 1E). MCEC lines expressed Cx43 and Cx45 and low levels of Cx37. Addition of labeled wtCx40-expressing cells to monolayers of untransfected MCEC resulted in a significant (P<0.05) increase in dye transfer compared with that achieved with MCEC expressing empty vector (Figure 1F). MCEC expressing Cx40T202S did not exhibit the same increase in dye coupling (Figure 1F).

The dye transfer and electric coupling data together suggest that the Cx40T202S mutant fails to form functional hemichannels but forms gap junctional channels that are permeable to electric, but not chemical, signals.

**Transgenic Mice Express Wild-Type or Mutant Cx40 Selectively in the Endothelium**

Transgenic mice were created to coexpress wtCx40 (Cx40Tg) or Cx40T202S (Cx40T202STg) constructs with the reporter enhanced green fluorescent protein (EGFP), under control of the endothelial-specific promoter Tie2 because neither protein would be distinguishable from endogenously expressed Cx40, using antibodies made against intracellular epitopes. mRNA for EGFP was expressed in the arteries of both transgenic strains (Figure 2A, top). Importantly, mRNA for the Cx40 or Cx40T202S transgenes was only expressed in the appropriate arterial samples, using primers which distinguished between the 2 Cx40 transgenes and endogenous Cx40 (Figure 2A, middle and bottom).

Protein expression of wtCx40 and Cx40T202S transgenes was verified by Western blotting of arterial homogenates and detection of EGFP (Figure 2B). Tissue specificity of transgene expression was confirmed by immunohistochemical detection of EGFP selectively in the endothelium of cremaster arteri-oles, mesenteric arteries (Figure 2C), basilar and renal vessels (data not shown).

**Body Weight, Blood Pressure, and Heart Rate Are Not Altered in Cx40T202STg Mice**

There were no significant differences found among the average body weights of wild-type, transgenic, and Cx40KO mice (Table 1). Blood pressure and heart rate of Cx40T202STg or Cx40Tg mice were also not significantly different from values recorded in wild-type mice (Table 1). In contrast, Cx40KO mice were hypertensive, as reported previously.19

**Expression of the Cx40T202S Transgene Does Not Eliminate Cx37 Expression**

To determine the effect of the Cx40T202S transgene on the oxerexpression of Cx40 on other endothelial Cxs, mesen-teric arteries from wild-type, Cx40T202STg, and Cx40Tg mice were probed using antibodies to Cx37, Cx40, and Cx43. Staining for Cx37 and Cx40 was found delineating endothelial cells in arteries from wild-type and transgenic mice, whereas Cx43 was not detected (Figure 3A). Quantification revealed a significant decrease in Cx37 expression in both transgenic strains, but no significant change to Cx40 (Figure 3B and 3C). Cx40 and Cx37 were absent from mesenteric arteries of Cx40KO mice (Figure 3A).

**Endothelial Cx40T202S Expression Enhances Myogenic Activation and Impairs EDH in Mesenteric Resistance Arteries**

We assessed the effect of transgene expression on active diam-eter (in the presence of CaCl₂) and the myogenic response of mesenteric resistance arteries. Mice used for these studies and for calculations of arterial stiffness were not significantly different in age (wild-type: 10.6±0.4 weeks; Cx40T202STg: 11.2±0.6 weeks; Cx40Tg: 10.1±0.5 weeks).

Active diameter of Cx40T202STg arteries was significantly less than wild-type (30–50 mm Hg) and Cx40Tg arteries (40–90 mm Hg; Figure 4A). In contrast, arteries of Cx40KO mice were significantly more relaxed than wild-type (50–90 mm Hg) and Cx40Tg (70–90 mm Hg; Figure 4A).

The myogenic response was activated at significantly lower pressures in Cx40T202STg arteries compared with wild-type, whereas activation of arteries from Cx40Tg mice occurred at significantly higher pressures (Figure 4B; Table 2). There was no significant difference between...
the maximum tone reached by arteries from wild-type and transgenic strains (Figure 4B; Table 2). Arteries from Cx40KO mice were less sensitive to pressure, and maximum tone achieved was significantly lower than wild-type (Figure 4B; Table 2).

Because gap junctions have been implicated in vasodilation attributable to EDH,29 we investigated the role of dilatory pathways in modulating myogenic responses. Elimination of nitric oxide, using the nitric oxide synthase inhibitor, L-NAME, in combination with the nitric oxide scavenger, hydroxocobalamin, increased the sensitivity of the myogenic response in all 4 genotypes and the maximum tone in wild-type and Cx40T202STg mice (Figure 4C–4F, Table 2). Because hydroxocobalamin can quench hydrogen sulfide, possible effects of this new signaling molecule could not be identified. Subsequent addition of TRAM-34 (IKCa antagonist) and UCL1684 (SKCa antagonist) to block EDH increased the myogenic sensitivity to pressure in wild-type and Cx40Tg mice but was without effect in Cx40T202STg or Cx40KO mice (Figure 4C–4F, Table 2).

Endothelial Cx40T202S Expression Increases Stiffness of Mesenteric Resistance Arteries

Given the enhanced myogenic activation of arteries from Cx40T202STg mice, we determined whether lifetime expression of Cx40T202S could cause mechanical changes in these vessels. Analysis of the passive diameter-pressure relationship showed no significant difference between arteries from wild-type and transgenic mice; however, passive lumen diameter of Cx40T202STg arteries was significantly narrower than Cx40Tg arteries at pressures >40 mm Hg (Figure 5A).

### Table 1. Weight and Cardiovascular Characteristics of Different Mouse Strains

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type (n=8)</th>
<th>Cx40T202STg (n=10)</th>
<th>Cx40Tg (n=8)</th>
<th>Cx40KO (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>27.0±0.6</td>
<td>25.5±1.2</td>
<td>25.7±1.3</td>
<td>25.6±0.4</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>110±2</td>
<td>112±2</td>
<td>110±3</td>
<td>128±2*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>81±2</td>
<td>81±2</td>
<td>82±2</td>
<td>93±2*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>660±10</td>
<td>650±14</td>
<td>703±19</td>
<td>645±18</td>
</tr>
</tbody>
</table>

*P<0.05 compared with wild type.

---

Figure 3. Expression of Cx40 transgenes does not abrogate Cx37 expression. A, Confocal images of mesenteric endothelium stained with antibodies to Cx37, Cx40, and Cx43 (green) and DAPI nuclear stain (red). Cx37 and Cx40 were expressed at the endothelial borders of wild-type (WT), Cx40T202STg, and Cx40Tg mice, but not in the endothelium of Cx40KO mice. Cx43 was not detected in the endothelium of any of these strains. B and C, Quantification of Cx staining; Cx37 expression was reduced in Cx40T202STg and Cx40Tg mice, compared with WT mice (B), Cx40 expression was unchanged (C). *P<0.05 vs WT; n=no. of mice.

Figure 4. Mesenteric arteries from wild-type (WT), Cx40T202STg, Cx40Tg, and Cx40KO mice respond differently to intraluminal pressure. A, Cx40T202STg (n=13) arteries were significantly more constricted than those of WT (n=13) and Cx40Tg (n=13) mice, whereas Cx40KO (n=11) arteries were significantly more dilated. B, The myogenic response was activated at lower pressures in Cx40T202STg arteries and at higher pressures in Cx40Tg arteries, compared with WT. Maximum constriction of Cx40KO arteries was smaller than in the other genotypes. C and D, In WT arteries (n=7), blockade of NO with L-NAME and hydroxocobalamin, and endothelium-derived hyperpolarization (EDH) with TRAM-34 (IKCa) and UCL1684 (SKCa) shifted activation to lower pressures. D, In Cx40T202STg arteries (n=6) arteries, blockade of NO shifted activation of the myogenic response to lower pressures, but EDH had no effect. E, Sensitivity of Cx40Tg (n=6) arteries was similar to that of WT arteries. F, Sensitivity of Cx40KO (n=6) arteries was similar to that of Cx40T202STg arteries. *P<0.05 compared with WT; †P<0.05 compared with Cx40Tg.
narrowing was not attributable to eutrophic or hypertrophic remodeling because media/lumen ratios were equal for all genotypes (Figure 5B). However, the strain–pressure relationship was significantly reduced in Cx40T202STg arteries compared with wild-type and Cx40Tg, suggesting reduced distensibility (Figure 5C). Subsequent analysis of the stress–pressure relationship showed no difference between the genotypes; however, analysis of the stress–strain relationship revealed a leftward shift in Cx40T202STg arteries (Figure 5D and 5E). When exponential curves were fitted to the stress–strain data from each artery, the mean coefficient value was significantly higher in Cx40T202STg arteries compared with wild-type and Cx40Tg arteries, indicating an increase in the wall component stiffness (Figure 5F).

Responses to Contracting and Dilating Agents
Arteries from Cx40Tg mice were significantly more sensitive to the vasodilator, calcitonin gene related peptide (CGRP), than wild-type or Cx40T202STg arteries (Figure II and Table I in the online-only Data Supplement). Although maximal relaxation to CGRP did not vary between the genotypes, the increased vascular tone of Cx40T202STg arteries resulted in significant residual tone in the presence of supramaximal concentrations of CGRP (Table I in the online-only Data Supplement).

There was no difference among the genotypes in the sensitivity to phenylephrine (PE) or the total vascular tone achieved at 70 mm Hg (Figure III in the online-only Data Supplement). However, the maximum constriction to PE was significantly greater in Cx40Tg arteries than in Cx40T202STg and wild-type arteries (Table I in the online-only Data Supplement).

Total vascular tone achieved at 70 mm Hg in the presence of αβ-methylene-ATP was also not significantly different among the genotypes; however, the magnitude of the constriction was significantly greater in Cx40Tg than in Cx40T202STg arteries, as was the rebound vasodilation (Figure IV in the online-only Data Supplement).

Discussion
Our data show for the first time that perturbations in endothelial Cx40 function cause significant effects on the myogenic response to intraluminal pressure and on arterial stiffness; vascular characteristics which are altered in cardiovascular disease. Consistent with these data, mice overexpressing Cx40 in the endothelium show myogenic responses, which are less sensitive to intraluminal pressure, and show no sign of arterial stiffening. These data have been obtained in mice in which we have introduced a mutant form of Cx40 into the endogenous Cx40 population. We have therefore also shown that a single-point mutation in endothelial Cx40 can produce significant alterations in cardiovascular function; a causal association previously suggested for only human Cx40 polymorphisms.

Table 2. Characteristics of the Myogenic Response in Different Mouse Strains

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type</th>
<th>Cx40T202STg</th>
<th>Cx40Tg</th>
<th>Cx40KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 control, mm Hg</td>
<td>42.9±1.2 (13)</td>
<td>33.8±1.2 (13) *</td>
<td>50.2±0.9 (13) *</td>
<td>52.9±3.9 (11) *</td>
</tr>
<tr>
<td>EC50 blocking NO, mm Hg</td>
<td>37.1±1.4 (7) †</td>
<td>28.4±1.2 (6) †</td>
<td>40.1±1.1 (6) †</td>
<td>46.7±2.5 (6) †</td>
</tr>
<tr>
<td>EC50 blocking NO + EDH, mm Hg</td>
<td>31.5±1.3 (7) ‡</td>
<td>28.5±1.5 (6)</td>
<td>35.6±0.8 (6) ‡</td>
<td>44.7±2.5(7)</td>
</tr>
<tr>
<td>Max tone (1-D/Dmax)</td>
<td>0.35±0.01 (13)</td>
<td>0.36±0.01 (13)</td>
<td>0.33±0.02 (13)</td>
<td>0.24±0.03 (13) *</td>
</tr>
<tr>
<td>Max tone (1-D/Dmax) blocking NO</td>
<td>0.40±0.01 (7) †</td>
<td>0.41±0.01 (6) †</td>
<td>0.35±0.02 (6)</td>
<td>0.29±0.03 (6)</td>
</tr>
<tr>
<td>Max tone (1-D/Dmax) blocking NO + EDH</td>
<td>0.41±0.01 (7)</td>
<td>0.39±0.01 (6)</td>
<td>0.36±0.02 (6)</td>
<td>0.29±0.03 (6)</td>
</tr>
</tbody>
</table>

EDH, endothelium-derived hyperpolarization.
*P<0.05 compared with wild-type; †P<0.05 compared with control conditions; ‡P<0.05 compared with blocking NO conditions.
To study endothelial Cx40, our goal was to avoid the coordinate downregulation of endothelial Cx37 seen after the absence of Cx40 in Cx40KO mice (present study).\textsuperscript{25,26} In line with this, our immunohistochemical studies showed that Cx37 was not lost from the endothelial cell membrane of Cx40T202STg mice, although expression was reduced to 60%. This interesting result may suggest that coordinate regulation of Cx37 and Cx40 results from commonality in their interaction partners in the multiprotein nexus, which is increasingly seen to control Cx trafficking, localization, and function.\textsuperscript{34} In support of this, a similar decrease in Cx37 expression was also found in the arteries of our Tg mice overexpressing Cx40. However, in spite of this commonality in effects on Cx37 expression, the effects of the wild-type and mutant Cx40T202S transgenes on arterial function and stiffness were always opposite. Therefore, we conclude that the arterial dysfunction, which we have observed in Cx40T202STg mice, can be attributed to effects on endothelial Cx40, without contribution from Cx37.

To understand how the Cx40T202S mutation affected arterial function, we conducted in vitro experiments in oocytes and endothelial cells to investigate both hemichannel and gap junctional function. Although the Cx40T202S mutant did not form functional hemichannels, experiments in paired oocytes demonstrated that the mutant was expressed in the cell membrane and could form electrically competent gap junctions with other mutant Cx40T202S molecules, as well as with wtCx40 or wtCx37 hemichannels. However, the Cx40T202S mutation did not act as a dominant negative to downregulate electric function of wtCx40 gap junctions or hemichannels, although such an effect was found on hemichannels formed with Cx38, the amphibian orthologue of Cx37. Nevertheless, the passage of larger molecules through mutant Cx40T202S gap junctions was impaired in endothelial cells. From these data, we conclude that the Cx40T202S mutant impairs the function of endothelial hemichannels, as well as chemical, but not electric coupling between endothelial cells, or through myoendothelial gap junctions.

In cardiovascular diseases and aging, the contractile properties of resistance arteries have been shown to be altered.\textsuperscript{32,33,35} For this reason we chose to examine the myogenic constriction of third order mesenteric arteries in our transgenic mouse strains. Our data demonstrate that selective impairment of endothelial Cx40 enhances activation of the myogenic response, whereas overexpression of Cx40 reduces sensitivity; both without effect on the maximal response. In contrast, arteries from Cx40KO mice exhibited both reduced sensitivity to pressure and lower maximum tone. As different models of cardiovascular disease demonstrate both increased and decreased contractility of arteries, measured using pressure myography,\textsuperscript{32,35} we suspect that the decreased contractility of Cx40KO arteries is likely attributable to 2 exogenous, but related, factors. First, these arteries encounter extremely high pressures in vivo,\textsuperscript{39} an effect that has been shown to cause a shift in myogenic activation to higher pressures.\textsuperscript{32} Second, Cx40KO arteries in vivo are chronically exposed to the potent vasoconstrictor, angiotensin II. A lack of this stimulus in the in vitro pressure myograph may be expected to reduce contractility.

Because the site of impairment in Cx40T202STg mice is endothelial, it was important that we assess whether altered endothelial dilator capacity was responsible for the enhanced myogenic activation. In mesenteric arteries of wild-type mice, we found that both endothelial nitric oxide production and EDH were significant modulators of the myogenic response in the absence of any agonist or flow-induced activation. This in itself was a surprising finding, suggesting that intraluminal pressure per se could stimulate the release of these vasodilatory factors. Consistent with the data obtained in wild-type arteries, nitric oxide and EDH were also important for desensitizing the myogenic response in pressurized arteries from our Cx40Tg mice, which overexpress endothelial Cx40. However, blockade of EDH had no effect on arteries from Cx40T202STg or Cx40KO mice, indicating that perturbation in endothelial Cx40 function completely abrogates this myogenically induced EDH activity.

Cx40 has been strongly implicated in the agonist-induced activation of EDH in small mesenteric arteries because endothelial loading of antibodies to Cx40, but not to Cx37 or Cx43, reduced EDH and immunoelectronmicroscopy-localized Cx40 to myoendothelial gap junctions.\textsuperscript{36} Given that our Cx40T202S mutant is capable of forming electrically competent gap junctions, it seems unlikely that the myogenically induced activation of EDH in response to increased intraluminal pressure, found in this study, could be initiated in the endothelium. In contrast, because the Cx40T202S mutation reduces chemical coupling through gap junctions, our results provide compelling evidence that EDH is activated by substances moving from the smooth muscle to the endothelium through myoendothelial gap junctions, after smooth muscle constriction (Figure 6). Such movement of a chemical mediator, such as calcium or IP\textsubscript{3}, through myoendothelial gap junctions, has been suggested previously to be responsible for the termination of agonist-induced vasoconstriction, attributable to activation of nitric oxide or EDH.\textsuperscript{37–41} Our data show that this chemical movement is mediated by gap junctions comprising endothelial Cx40, and that the response can be activated physiologically by raising intraluminal pressure. The lack of effect on nitric oxide in our Cx40T202STg mice suggests that basal release of nitric oxide in pressurized small mesenteric arteries does not involve such a mechanism.

In suggesting this role, we acknowledge that endothelial Cx40 hemichannels could form myoendothelial gap junctions with any of the 3 potentially expressed smooth muscle Cxs (Cx37, Cx43, Cx45).\textsuperscript{42–44} and that the properties of these Cxs may differentially affect the chemical permeability of the endothelial connexons composed of Cx40T202S.\textsuperscript{7} Although our findings in Cx40T202STg mice are consistent with impaired myoendothelial feedback, they do not allow us to specify which of the potential heterotypic gap junctions may be involved.

The observation of enhanced constriction after lifelong alteration to endothelial Cx function in arteries of our Cx40T202STg mice led us to investigate whether this
stress could lead to changes in artery structure, as it occurs in hypertension and aging. Although pressure-diameter relationships of mesenteric arteries were not significantly different between wild-type and Cx40 overexpressing transgenic strains, Cx40T202S mutant arteries exhibited increased contractility, reduced distensibility, and increased component stiffness of the vascular wall. We further show that increased contractility at physiological intraluminal pressures in these vessels leads to reduction in the maximal vasodilation evoked by endogenous vasodilators, without change in sensitivity. Vasodilation after vasoconstriction evoked by the neural agonist, αβ-methylene-ATP, was also attenuated. Our data therefore provide new evidence that endothelial hemichannels, or chemical coupling via endothelial gap junctions can result in arterial stiffening and attenuated responses to endogenous vasodilators.

Although polymorphisms in the human Cx40 promoter region have been shown to reduce Cx40 mRNA levels in atria and correlate with the incidence of atrial fibrillation, only 1 study has investigated a possible relationship with coronary artery disease; however, no association was found. To our knowledge, this is the first report of a causal link between impairment of endothelial Cx40, as has been seen in hypertension and aging, and the arterial stiffening and dysfunction observed in these conditions. Importantly, this vascular dysfunction has been induced by a single-point mutation in an extracellular loop of Cx40, existing as a polymorphism in an endogenous endothelial population of Cx40 molecules.

The results of the present study demonstrate that chemical coupling by endothelial Cx40 is necessary for basal EDH, an effect recently shown to depend on myoendothelial feedback. The chronic impairment of this vasodilatory function in Cx40T202STg mice causes enhanced myogenic activation and likely contributes to the increased stiffness found in mesenteric resistance arteries, although impairment to endothelial hemichannels, where Cx37 is expressed, could also contribute to the latter phenomenon. To our knowledge, this is the first report of a causal link between impairment of endothelial Cx40, as has been seen in hypertension and aging, and the arterial stiffening and dysfunction observed in these conditions. Importantly, this vascular dysfunction has been induced by a single-point mutation in an extracellular loop of Cx40, existing as a polymorphism in an endogenous endothelial population of Cx40 molecules.

Acknowledgments
We thank Helen Taylor and Mohammad Alkhrayef for technical assistance.

Sources of Funding
We acknowledge support from the National Health and Medical Research Council (471421).

Disclosures
None.

References
2. Severs NJ, Rothery S, Dupont E, Coppen SR, Yeh HI, Ko YS, Matsumiya T, Kaba R, Halliday D. Immunocytochemical analysis of connexin
common connexin-40 gene promoter variant affects connexin-40 expression in human atria and is associated with atrial fibrillation / clinical perspective. Circ Arrhythm Electrophysiol. 2011;4:87–93


Significance

Mutant forms of Cx40 are known to exist in the human population, but their specific effect on arteries is unknown. To our knowledge, this is the first study to show that the presence of a mutant Cx40 in the vascular endothelium can cause changes to artery function that are associated with cardiovascular disease.
Polymorphism in Endothelial Connexin40 Enhances Sensitivity to Intraluminal Pressure and Increases Arterial Stiffness


Arterioscler Thromb Vasc Biol. 2013;33:962-970; originally published online March 7, 2013; doi: 10.1161/ATVBAHA.112.300957

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/5/962

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/03/07/ATVBAHA.112.300957.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
## Supplement Material

### Supplementary Table I. Characteristics of responses to vasodilators and vasoconstrictors

<table>
<thead>
<tr>
<th>Curve</th>
<th>Parameter</th>
<th>Wildtype (6)</th>
<th>Cx40Tg (6)</th>
<th>Cx40T202STg (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Supp Fig. 2B</strong></td>
<td>CGRP Vascular tone</td>
<td>EC\textsubscript{50}</td>
<td>-8.5 ± 0.1</td>
<td>-9.3 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>1-(D/DMax)</td>
<td>Min</td>
<td>0.03 ± 0.02</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td><strong>Supp Fig. 2C</strong></td>
<td>CGRP relaxation (%)</td>
<td>EC\textsubscript{50}</td>
<td>-8.4 ± 0.1</td>
<td>-9.4 ± 0.3*</td>
</tr>
<tr>
<td></td>
<td>((D-D\textsubscript{0})/(DMax-D\textsubscript{0}))x100</td>
<td>Max</td>
<td>84 ± 9</td>
<td>96 ± 13</td>
</tr>
<tr>
<td><strong>Supp Fig. 3B</strong></td>
<td>PE Vascular tone</td>
<td>EC\textsubscript{50}</td>
<td>-6.2 ± 0.1</td>
<td>-6.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1-(D/DMax)</td>
<td>Max</td>
<td>0.67 ± 0.01</td>
<td>0.69 ± 0.01</td>
</tr>
<tr>
<td><strong>Supp Fig. 3C</strong></td>
<td>PE Constriction</td>
<td>EC\textsubscript{50}</td>
<td>-6.2 ± 0.1</td>
<td>-6.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>((D-D\textsubscript{0})/DMax) x100</td>
<td>Max</td>
<td>43.7 ± 1.5</td>
<td>53.1 ± 1.6*</td>
</tr>
<tr>
<td>DMax (µm)</td>
<td></td>
<td>70mmHg</td>
<td>177 ± 8</td>
<td>182 ± 8</td>
</tr>
</tbody>
</table>

*P<0.05, compared to Wildtype; †P<0.05 relative to Cx40Tg; number of arteries/mice in parentheses.
Supplementary Figures

Figure I. Location of T202S mutation and design of transgene.

(A) The mutation is located in the second extracellular loop in the region targeted by the inhibitory mimetic peptide 40Gap27. (B) Transgene constructs consist of the endothelium specific promoter Tie2, followed by the mutant wildtype Cx40 (I) or Cx40T202S (II), an IRES sequence, reporter EGFP, poly A tail, and Tie2 enhancer segment.

Figure II. Responses of small mesenteric arteries to the vasodilator CGRP.

(A) Representative traces showing concentration-dependent vasodilation to CGRP of arteries from wildtype (WT; n=6), Cx40Tg (n=6) and Cx40T202STg (n=7) mice. (B) Vascular tone decreased in a concentration-dependent manner in all genotypes, however the minimum tone achieved was higher in Cx40T202STg arteries. (C) Arteries from Cx40Tg mice were more sensitive to CGRP than arteries from WT or Cx40T202STg mice. See Supplementary Table 1 for details.

Figure III. Responses of small mesenteric arteries to the vasoconstrictor, phenylephrine (PE).

(A) Representative traces of arteries from wildtype (WT; n=6), Cx40Tg (n=6) and Cx40T202STg (n=7) mice, showing concentration-dependent vasoconstriction to PE. (B) Vascular tone at 70 mmHg, in the presence of supramaximal PE (30 µM), was not significantly different amongst the genotypes. (C) Maximal vasoconstriction to PE was significantly greater in arteries from Cx40Tg, compared to WT or Cx40T202STg mice. See Supplementary Table 1 for details.

Figure IV. Responses of small mesenteric arteries to the vasoconstrictor, αβ-methylene-ATP.

(A) Representative traces of arteries from wildtype (WT; n=6), Cx40Tg (n=6) and Cx40T202STg (n=7) mice, showing vasoconstriction to αβ-methylene-ATP and subsequent vasodilation. (B) Vascular tone at 70 mmHg, in the presence of αβ-methylene-ATP (1 µM), was not significantly different amongst the genotypes. (C, D) The maximum amplitude of the αβ-methylene-ATP-induced vasoconstriction and area under the curve was significantly less for Cx40T202STg arteries than for Cx40Tg arteries. (D) Vasodilation following αβ-methylene-ATP-induced vasoconstriction was also significantly less for Cx40T202STg arteries than for Cx40Tg arteries. †P<0.05 relative to Cx40Tg.
A

Supplementary Figure I

**A**

Cx40T202S

Extracellular Plasma Membrane

Mutation in EL2 at T202

Cytoplasm

N-Terminus

C-Terminus

**B**

Transgene construct

I. Cx40Tg

Tie 2 Promoter → Cx40 → IRES → EGFP

II. Cx40T202STg

Tie 2 Promoter → Cx40T202S → IRES → EGFP
Supplementary Figure II

A

Active Diameter (µm) vs. Log [CGRP]

WT

Cx40Tg

Cx40T202STg

Time (min)

B

Vascular tone (L/D/Max) vs. Log [CGRP]

WT

Cx40Tg

Cx40T202STg

C

% Relaxation vs. Log [CGRP]

WT

Cx40Tg

Cx40T202STg
Supplementary Figure III

A

![Graph A](image)

Active Diameter (µm) vs. Log [PE]

WT

Cx40Tg

Cx40T202STg

Time (min)

B

![Graph B](image)

Vascular tone (1-D/DMax) vs. Log [PE]

WT

Cx40Tg

Cx40T202STg

C

Constriction amplitude ([D0 · D]/DMax) * 100 vs. Log [PE]

WT

Cx40Tg

Cx40T202STg
Supplementary Figure IV

A

Log [αβ-methylene-ATP]

Area Under Curve: Dilation

Area Under Curve: Constriction

WT

Cx40Tg

Cx40T202STg

Active Diameter (µm)

Active Diameter (µm)

Active Diameter (µm)

Time (min)

B

Vascular tone (1-D/DMax)

WT  Cx40Tg  Cx40T202STg

C

Constriction amplitude (1-D/DMax)*100

WT  Cx40Tg  Cx40T202STg

D

Constriction area under curve (µm.s)

WT  Cx40Tg  Cx40T202STg

E

Dilation area under curve (µm.s)

WT  Cx40Tg  Cx40T202STg
Supplementary Figure 1

A

Cx40T202S

Mutation in EL2 at T202

Extracellular Plasma Membrane

M1 M2 M3 M4

Cytoplasm

N-Terminus

C-Terminus

B

Transgene construct

I. Cx40Tg

Tie 2 Promoter → Cx40 → IRES → EGFP

II. Cx40T202STg

Tie 2 Promoter → Cx40T202S → IRES → EGFP
Supplementary Figure 2

A

-10 -9.5 -9 -8.5 -8 -7.5 Log [CGRP]

WT

Cx40Tg

Cx40T202STg

Active Diameter (µm)

Time (min)

B

[Graph showing vascular tone (L-D/Max) vs. Log [CGRP] for WT, Cx40Tg, and Cx40T202STg]

C

% Relaxation vs. Log [CGRP] for WT, Cx40Tg, and Cx40T202STg
Supplementary Figure 3

A

Log [PE]

WT

Cx40Tg

Cx40T202STg

Active Diameter (µm)

Active Diameter (µm)

Active Diameter (µm)

Time (min)

B

Vascular tone (1-D/DMax)

Log [PE]

WT

Cx40Tg

Cx40T202STg

C

Constriction amplitude (ID0/DMax)*100

Log [PE]
Supplementary Figure 4

A

Log [αβ-methylene-ATP]

Area Under Curve: Dilation

Area Under Curve: Constriction

WT

Cx40Tg

Cx40T202STg

Active Diameter (µm)

Active Diameter (µm)

Active Diameter (µm)

Time (min)

B

Vascular tone (1-D/DMax)

WT

Cx40Tg

Cx40T202STg

C

Constriction amplitude (D0-D)/(DMax)*100

WT

Cx40Tg

Cx40T202STg

D

Constriction area under curve (µm²)

WT

Cx40Tg

Cx40T202STg

E

Dilation area under curve (µm².s)

WT

Cx40Tg

Cx40T202STg
Materials and Methods

Ethics Statement

All experimental animal procedures were carried out in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes of the National Health and Medical Research Council of Australia. Protocols were approved by the Animal Experimentation Ethics Committee of the Australian National University (JMB.33.07; JNS.43.08; A2011/72). All experiments were carried out on male mice aged 8 to 12 weeks. Cx40KO breeding pairs were kindly provided by Dr A.M. Simon (University of Arizona).

Development of Cx40 constructs

Two constructs containing either Cx40 or a mutant form of Cx40 (Cx40T202S; see description below), in which threonine was replaced with serine at position 202 (Supplementary Figure 1A) were developed. This mutation was chosen for its location in the centre of the conserved peptide sequence SRPTEK, previously shown to inhibit gap junction function.1-2 Cx40 genes were expressed with EGFP as a reporter gene, through the use of an internal ribosome entry site (IRES) which allows the expression of a bicistronic mRNA and the production of the two independent proteins, Cx40 and EGFP, from the same promoter.

Cx40 (or mutant Cx40)-IRES-EGFP-polyA: The basic Cx40 plasmid was constructed in pBluescriptKSII (pBKSI) as follows:

A full-length 1.3kb BamH I/Nae I Cx40 fragment 3 was cloned into the BamH I site of pBKSI and an IRES-EGFP reporter was added from pIRES2-EGFP (Clontech), in addition to a BamH I/Not I fragment of the human growth hormone polyadenylation signal (kind gift from Professor Jefferey Whitsett, Cincinnati Childrens Hospital Center, Cincinnati, USA).4 This vector was called pBKSIICx40-IRES-EGFP-polyA ready to be coupled to the endothelial specific promoter, Tie2, as described below.

An equivalent Cx40T202S-IRES-EGFP-polyA construct was generated after synthesis of a BspE I/Xcm I fragment of Cx40 with a single base pair change (A to T) at coding position 604 (Genscript, USA). The new synthetic Cx40T202S fragment was inserted into the Cx40-IRES-EGFP-PolyA construct after digestion with BspE I/Xcm I to remove the native Cx40 fragment.

RNA expression vectors: The entire Cx40-IRES-EGFP (Eag I-Eag I) or Cx40 only (Eag I-Cla I) fragments were subcloned into the Eag I-Cla I or Eag I sites, respectively, of pSGEM (Promega). This vector contains both T7 and SP6 RNA polymerase promoter sites and the genes were oriented in the 5’UT–Cx40–IRES–EGFP–3’UT direction to generate RNA for the oocyte hemichannel and gap junction experiments. Mutant Cx40T202S plasmids were constructed in a similar manner.

Transgenic Mice

Transgenic mice were created to express either Cx40 (Cx40Tg) or Cx40T202S, controlled by the endothelium specific promoter Tie2 (Supplementary Figure 1B). The constructs used to create transgenic mice consisted of the following elements in order; Tie2 promoter, Cx40 or Cx40T202S, an IRES for bicistronic expression of EGFP, a human growth hormone polyadenylation signal, Tie2 enhancer (Supplementary Figure 1B).
**Tie2-Cx40-IRES-EGFP-polyA-Enhancer construct:** The plasmid pHHSDKXXK, containing the Tie2 promoter and a minimal enhancer fragment, as well as the plasmid pg50-2.11 containing the full length Tie2 enhancer, were generous gifts from Professor Tom Sato, The University of Texas, Southwestern Medical Center at Dallas, Texas, USA. A 2kb Tie2 promoter fragment was recovered from pHHSDKXXK by Hind III digestion. This was inserted into pBKSII using Hind III and BamH I sites inside the multiple cloning site (with loss of the BamH I restriction site) to give pBKSII{Tie2}.

Digestion of pBKSII{Tie2} with Cla I, followed by blunting of the Cla I site, and partial digestion with Bcl I allowed recovery of the Tie2 fragment. This was ligated into the Sac II digested, blunted, then BamH I-digested backbone fragment of the Cx40-IRES EGFP-polyA construct described above. This placed the Tie2 promoter in a position to drive the expression of both Cx40 and EGFP.

The Tie2-Cx40-IRES-EGFP-polyA plasmid was linearised by digestion with Xho I, blunted, and ligated to the 10kb Tie2 enhancer fragment from Nae I and Sal I-digestion of pg50-2.11.5 The final construct now contained the enhancer in the forward orientation after the polyadenylation signal. Sequencing was performed at each step to confirm the presence, fidelity, and orientation of construct components.

**Tie2-Cx40T202S-IRES-EGFP-polyA-Enhancer construct:** The Tie2-Cx40T202S-IRES-EGFP-polyA-Enhancer construct was then created in a similar manner by the addition of the Tie2 promoter and Cx40 enhancer fragments to the Cx40T202S-IRES-EGFP-polyA plasmid created above.

**Tie2-Cx40 -EGFP and Tie2-Cx40T202S-EGFP mice:** The final constructs were digested with Pvu I and BssH II and gel purified to remove vector sequence prior to use in the generation of transgenic mice. Transgenic C57BL/6 mice were produced by pronuclear injection of the 19kb construct (TASQ, University of Queensland, Australia). Transgenic founders were identified by PCR, mated and their progeny were analyzed for EGFP expression. The founders whose progeny had the highest EGFP expression were used to set up single mouse colonies for each genotype.

**Genotyping and mRNA Expression of Transgenes**

The Cx40 and Cx40T202S transgenes were detected with specific primers for each transgene: forward primers, 5'- GTC-AAC-TGT-TAT-TGG-TTG-AGG-CCC-A-3' (Cx40) and 5'-GTC-AAC-TGT-TAT-TGG-TTG-AGG-CCC-T-3' (Cx40T202S), located in the protein coding region and a common reverse primer, 5'-GGG-CGG-ATC-TTC-AAT-CAG-GCT-AT-3', located in the non-coding region adjacent to the IRES component of the transgene. Using these primers, a 618bp fragment was amplified in a PCR reaction comprising: 95°C for 3 min, then 37 cycles of 95°C for 20 s, 70°C for 20 s, and 72°C for 60 s, followed by 72°C for 3 min. The EGFP component of the transgenes was detected using the forward primer, 5'-CGA-CGT-AAA-CGG-CCA-CAG-GTT-CG-G-3' and reverse primer, 5'-GCT-TTA-CTT-GTA-CAC-GTC-GTC-CAT-3', which generated a 661bp fragment in a PCR reaction comprising 95°C for 3 min, then 35 cycles of 95°C for 10 s, 67°C for 10 s, 72°C for 30 s, followed by 72°C for 3 min (Corbett thermal sequencer).

Mice were anaesthetised with isoflurane and decapitated. Aortas were removed into cold phosphate buffered saline (PBS), isolated from surrounding fatty tissues, frozen in liquid nitrogen and pulverised with a chilled pestle and mortar. RNA was extracted using an RNaseasy minikit (Qiagen) according to the manufacturer’s instructions and
any contaminating genomic DNA removed by incubation with RNase free DNase (Qiagen) before reverse transcription of the RNA using Superscript RTII (Invitrogen) and oligoDT. Expression of mRNA was determined for Cx40Tg, Cx40T202STg and EGFP components of transgenes using PCR. Comparable reactions without reverse transcriptase controlled for any residual genomic DNA.

**Western Blotting**

Frozen aortas, prepared as above, were ground in sample buffer (37 mM TRIS, 0.5% lithium dodecysulphate, 2.5% glycerol, 0.13 mM EDTA, 0.06 mM SERVA blue G250, 0.04 mM phenol red, 50 mM DTT, 1X miniComplete Protease Inhibitor Cocktail [Roche], pH8.5), then heated at 70°C for 10 min, before being stored at -20°C until analysis. Proteins were separated by electrophoresis on NuPAGE® 4-12% BIS-TRIS gradient gels and transferred onto PVDF membranes, according to manufacturer’s protocols (Invitrogen).

Membranes were blocked (PBS, 5% bovine serum albumen, BSA, 0.05% Tween-20) for 2 h at room temperature, then probed for 16 h at 4°C with anti-GFP monoclonal antibodies (1:5000; kindly supplied by Jan Elliot, Research School of Biology, Australian National University) and subsequently for 2 h with horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies (1:5000; Upstate, Millipore). HRP was detected using Immobilon Western chemiluminescent substrate according to the manufacturer’s protocol (Millipore) and visualised on a LAS1000 system (Fujifilm). The EGFP +ve control was from a mouse ubiquitously expressing an EGFP tagged with a nuclear localization signal (NLS) and therefore has a slightly higher molecular weight (Matthaei unpublished).

**Immunohistochemistry**

**EGFP detection**

*Basilars artery, kidney, and mesenteric arteries*: Mice were anaesthetised with isoflurane and decapitated. Tissues were removed immediately and placed in ice cold PBS where they were removed from surrounding fatty and connective tissue. Tissues were then immersion fixed in 2% paraformaldehyde (0.1M sodium phosphate buffer) for 10 minutes, washed in PBS and then cryoprotected in 30% sucrose in PBS overnight. Tissues were embedded in Tissue Tek OCT mounting medium, frozen and cut into 30µm sections using a cryostat.

*Cremaster arterioles*: Mice were anaesthetised (1mg/kg medetomidine, 10mg/kg midazolam, and 0.1mg/kg Fentanyl, i.p.) before tracheotomy and jugular vein cannulation for continuous anaesthetic administration (0.2mg/h medetomidine, 0.2mg/h midazolam, 0.002mg/h fentanyl). Mice were warmed on a heating pad with a chamber custom built for intravital microscopy and their right cremaster muscle was cut open and spread over a coverslip. The cremaster muscle was continuously superfused (3ml/min) with Krebs solution (mmol/L): NaCl 118.4; KCl 3.8; NaHCO3 25; KH2PO4 1.2; CaCl2.2H2O 2.5; MgSO4 1.2 gassed with 5% CO2 in N2, pH 7.4, 34°C. The muscle was then removed, fixed in 2% paraformaldehyde and cryosections prepared as above.

All cryosections were preincubated in PBS containing 2% BSA, 0.2% TritonX-100, 0.04% sodium azide for 30 min before incubation for 24 h in rabbit anti-GFP (1:1000, Invitrogen) antibody. Primary antibodies were detected with Alexa 488 donkey anti-rabbit antibodies (1:400; Invitrogen). Finally, sections were stained with 0.01%
Pontamine Sky Blue dye for 3 min, to shift internal elastic lamina autofluorescence to a longer wavelength, then mounted in buffered glycerol.

Connexin detection

Mice were anaesthetised (25mg/kg ketamine and 5mg/kg xylazine, i.p.) before perfusion with saline, containing 0.1% BSA, 0.1% NaNO₃, 5 U/ml heparin at 60mmHg, followed by 2% paraformaldehyde in 0.1M sodium phosphate buffer, containing 0.1% NaNO₃. Primary mesenteric arteries were removed, cut open, pinned flat with the endothelium uppermost, washed in PBS and preincubated in PBS containing 2% BSA, 0.2% TritonX-100, 0.04% sodium azide for 30 min, before incubation for 48h with sheep antibodies against Cx37 (1:100) or Cx40 (1:100⁶⁻⁷) or rabbit antibodies against Cx43 (1:100, Invitrogen, Mulgrave, Victoria, Australia). Primary antibodies were detected with Cy3 conjugated donkey anti-sheep (1:400) or Cy3 donkey anti-rabbit immunoglobulin (1:400, Jackson ImmunoResearch Laboratories Inc. West Grove, Pennsylvania, USA) diluted in PBS containing 0.01% TritonX-100. Nuclei were stained with 15 µM DAPI in PBS and wholemounts were mounted on slides in buffered glycerol.

Image acquisition and analysis

Image series (0.3µm intervals) of all stained tissues were taken with a Leica SP2 confocal scanning microscope. For each antibody, the same acquisition settings were used to collect images from all genotypes, so that the degree of staining could be compared amongst the different mouse strains. Each image series was projected to a single image and three such composite images were obtained from each animal to provide an average/animal. Composite images were quantified for Cx staining using the thresholding function of Image J (National Centre for Biotechnology Information) and the amount of Cx staining was expressed per endothelial cell or endothelial cell perimeter.

Electrophysiology of Xenopus Oocytes expressing different combinations of wt Cx40 and 37 and mutant Cx40

Hemichannel activity: Oocytes were isolated using a previously published method⁸ and separated into two groups based on their endogenous (Cx38) current.⁹⁻¹¹ Endogenous current was measured in native oocytes, voltage clamped at -30mV and subjected to 1s voltage steps from -140 to 40mV, in 20mV increments (GeneClamp 500B, Axon Instruments, CA), before and after superfusion for 10 minutes with a divalent free solution (in mM: 96 NaCl, 2.5 KCl, 10 HEPES, 10 HEDTA to chelate residual divalents) to induce opening of hemichannels. Oocytes were then injected with 20ng of RNA transcribed in vitro from the psGEM vector, containing either Cx40-IRES-EGFP or Cx40T202S- IRES-EGFP, and allowed to express for two days before current measurements were made again, using the same protocol. Experiments were replicated on multiple oocytes obtained from at least two different donor females to ensure the reproducibility of the data and rule out individual variations. Each set of oocytes was collected in different weeks.

Gap junction activity: Gap junctional conductances between paired oocytes were measured, as previously described.¹² Isolated oocytes were injected with a mixture of antisense RNA (3-5ng) to suppress endogenous Cx38, ~24 hours prior to injection of cRNA (23-46ng) for Cx40, Cx40T202S or Cx37. Oocytes were allowed to express
injected transgenes for one day, before vitelline membrane removal and pairing in agar wells. After further incubation for 1-2 days, junctional conductance was measured in oocyte pairs. Both cells were clamped to -20mV, and one oocyte subjected to a 500ms -10mV prepulse, followed by 5s voltage steps from -120 to 80mV in 10-20mV increments. Junctional conductances were determined between oocyte pairs expressing the following Cx proteins: (Oocyte 1: Oocyte2) Cx40:Cx40, Cx40T202S:Cx40T202S, Cx40:Cx40T202S, Cx40:Cx40 + Cx40T202S, Cx37:Cx37, Cx37:Cx40T202S, Cx37:Cx37+Cx40T202S. In cases where a single Connexin type was expressed, 46ng of cRNA was injected. In cases where two connexin types were expressed, 23ng of each cRNA were mixed and injected. Junctional conductances of oocytes expressing Cx40T202S alone, or in combination with Cx40 or Cx37, were normalised to average conductances of either wildtype Cx40, or wildtype Cx37 pairs, respectively.

Subcloning Cx40 constructs and Generation of Stable Cell lines
For in vitro analysis the coding sequence for the wildtype and Cx40T202S mutant of murine Cx40 were subcloned into the EcoRV cloning site of the pcDNA3-neo mammalian expression vector (Invitrogen). Resulting constructs were verified by automated DNA sequencing. Expression of wildtype Cx40 or the Cx40T202S mutant in mouse cardiac endothelial cells (MCEC) was performed by transfection with cationic lipids (Lipofectamine 2000 reagent; Invitrogen), according to the manufacturer’s instructions. Stable transfection was achieved using Geneticin (800 μg/mL) as the selectable marker. Transfection with empty vector served as the control. Stably transfected MCEC were grown at 37°C in an atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (vol/vol) fetal bovine serum, 100U/ml penicillin/streptomycin, 2 mM glutamine and 10 mM HEPES.

Expression of wildtype Cx40 and Cx40T202S constructs was confirmed via immunoblotting. After rinsing MCEC monolayers with PBS, proteins were extracted using cold RIPA buffer (50 mM Tris-HCl, pH7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, protease inhibitor). Lysates were incubated on ice for 30 minutes, sonicated and clarified by centrifugation (16,000xg for 10 min). Aliquots (30-50 μg) were separated by SDS-PAGE on (8-15%) gradient polyacrylamide gels. Following transfer to PVDF membranes, primary antibodies, Cx40 (C-terminal; Alpha Diagnostic International) and α-tubulin (Santa-Cruz), were diluted in blocking buffer (TBS-T; 10 mM Tris (pH8.0), 150 mM NaCl, 0.1% (v/v) Tween-20; with 5% (w/v) non-fat milk powder) and incubated with membranes overnight at 4°C. Antibody binding was detected with HRP-conjugated secondary antibodies (1:2000 and 1:5000 respectively, Dako Cytomation, Dako Australia). Membranes were washed, exposed to chemiluminescent substrate (Perkin Elmer, Melbourne, Australia) and X-ray film used to capture images of the resulting bands.

Cell Parachute Assay of endothelial cells for chemical coupling
Gap junction coupling was assessed via the transit of fluorogenic esters from labelled to unlabelled cells (parachute assay) as previous described¹³ with modifications. Growth media was aspirated from monolayers of MCEC transfected with vectors encoding no protein (empty), wildtype Cx40 or the Cx40T202S mutant, and cells washed twice with PBS. MCEC were incubated with the gap junctional permeable ester (calcein-AM, 10 μM; Invitrogen) for 30 min at 37°C in serum-free media. Co-labelling with the membrane dye Dil (0.2 μM, Invitrogen) enabled
quantification of labelled cells added to co-cultured monolayers. Cells were washed three times with PBS, harvested using trypsin and suspended at 2x10^5 cells/mL in full growth media. Confluent monolayers of unlabelled MCEC in 6 well plates were the recipient cells for this assay. Media was aspirated and recipient MCEC washed with PBS. Labelled MCEC were added at a ratio of 1:4 with recipient cells and left for 16 hours for dye transfer to occur. At the end of this period cells were harvested using Trypsin-EDTA and analysed for dye transmission by flow cytometry. Gap junction coupling was determined by quantifying the calcein-labelled, Dil negative cells which could only have arisen from dye transfer between labelled and unlabelled cells. For analysis, 5000 pre-labelled cells (Dil positive) were captured per well and the number of calcein-labelled (Dil negative) events determined. Unlabelled and dual-labelled cells served as positive and negative controls for analysis.

**Pressure myography**

Mice were anaesthetised with isoflurane, decapitated and 3rd order mesenteric collateral arteries removed into ice cold calcium-free physiological salt solution (Ca-free PSS) containing; 120 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 2 mM MgSO₄, 1 mM NaH₂PO₄, and 11 mM glucose. Arteries were cannulated with glass micropipettes and pressurised to 50 mmHg (Living Systems Instruments, Burlington, VT, USA) and stretched by 33% to regain their original length, while superfused at 37°C with PSS containing 2 mM CaCl₂, gassed with 5% CO₂ in air. The mean length of arteries was not significantly different between the different genotypes before stretch (Wildtype: 849±76 µm, Cx40T202STg: 717±49 µm, Cx40Tg: 753±44 µm) or after stretch (Wildtype: 1285±118 µm, Cx40T202STg: 1094±84 µm, Cx40Tg: 1126±59 µm).

After equilibration at 50 mmHg for 1 h, the active diameter in the presence of CaCl₂ was determined at successive pressure steps of 10, 30, 40, 50, 70, and 90 mmHg. Pressure changes were made after vessel diameter stabilised (~7 min). L-NAME (100µM) and hydroxocobalamin (100µM; 30 min) were used to block nitric oxide, while TRAM-34 (1µM) and UCL1684 (1µM; 30 min) in combination were used to block EDH (IKCa and SKCa channels, respectively). Passive lumen diameter and wall thickness were measured in Ca-free PSS containing 10mM EGTA, for the same pressure steps.

For each pressure, vascular tone was calculated as 1- (D/DMax) where D=active lumen diameter and DMax=passive lumen diameter. Myogenic activation and maximum tone were determined by EC₅₀ values and maxima obtained from sigmoidal regression fit of vascular tone versus pressure data.

The mean ages of mice used in this and the following Section were not significantly different amongst the genotypes (Wildtype: 10.6±0.4 weeks, Cx40T202STg: 11.2±0.6 weeks, Cx40Tg: 10.1±0.5 weeks).

In separate experiments, responses to vasodilating and vasocontracting agents, which would normally be released on to these vessels from perivascular sensory and sympathetic nerves, were tested. Experiments were conducted at an intraluminal pressure of 70 mmHg to mimic pressures experienced in these resistance vessels in vivo. Against this level of myogenic tone, the effects of the bath-applied vasodilator, CGRP and vasoconstrictors, αβ-methylene-ATP and phenylephrine (PE), were tested. Responses were assessed after vessel diameter had stabilised after
exposure to the agent (~5 min). Only a single supramaximal concentration of αβ-methylene-ATP (1 µM) was tested due to rapid desensitisation of P2X receptors.14

Passive artery characteristics

Artery stiffness was assessed using passive diameter and wall thickness measurements. Circumferential wall strain was calculated for each pressure, using the equation \( \frac{(D-D_{10\text{mmHg}})}{D_{10\text{mmHg}}} \) and wall stress was calculated using the equation \( \frac{(P \times D)}{2WT} \), where \( D= \) passive diameter at pressure, \( P; D_{10\text{mmHg}}= \) passive diameter at 10mmHg, and \( WT= \) wall thickness. The β-coefficient indicator of vessel stiffness was calculated for each artery after fitting the stress-strain data to the exponential regression \( y=ae^{bx} \).

Blood Pressure

Blood pressure and heart rate were measured using tail cuff plethysmography on conscious, restrained mice. Mean blood pressure for individual animals was the average of measurements over 6 days, following 5 days acclimitisation to the apparatus.

Statistics

Statistical analyses and regression were performed using Graph-Pad prism software (Version 4). Groups were compared using one-way or two-way ANOVA, where appropriate, followed by Bonferonni’s or Tukey’s post-hoc tests. All data are expressed as means ± SEM. Differences were considered significant at \( P<0.05 \).
References


