Defective Cx40 Maintains Cx37 Expression but Intact Cx40 Is Crucial for Conducted Dilations Irrespective of Hypertension

Alexander Jobs, Kjestine Schmidt, Volker J. Schmidt, Indra Lübkemeier, Toon A.B. van Veen, Armin Kurtz, Klaus Willecke, Cor de Wit

Abstract—The gap junction channel protein connexin40 (Cx40) is crucial in vascular and renal physiology, because Cx40-deficient mice exhibit impaired conduction of endothelium-dependent dilations and pronounced hypertension. The latter precludes mechanistic insights into the role of endothelial Cx40, because long-lasting hypertension itself may affect conduction and Cx expression. We aimed to identify endothelial Cx40 functions, their dependency on the conductive capability, and to separate these from hypertension-related alterations. We assessed conduction and Cx expression in mice with cell type–specific deletion of Cx40 and in mice expressing a defective Cx40 (Cx40A96S) identified in humans, which forms nonconducting gap junction channels. Confined arteriolar stimulation with acetylcholine or bradykinin elicited local dilations that conducted upstream without attenuation of the amplitude for distances up to 1.2-mm in controls with a floxed Cx40 gene (Cx40^fl/fl). Conducted responses in hypertensive animals devoid of Cx40 in renin-producing cells were unaltered but remote dilations were reduced in normotensive animals deficient for Cx40 in endothelial cells (Cx40^fl/fl).: Tie2-Cre). Surprisingly, Cx37 expression was undetectable by immunostaining in arteriolar endothelium only in Cx40^fl/fl:: Tie2-Cre; however, transcriptional activity of Cx37 in the cremaster was comparable with Cx40^fl/fl controls. Cx40A96S mice were hypertensive with preserved expression of Cx40 and Cx37. Nevertheless, conducted responses were blunted. We conclude that endothelial Cx40 is necessary to support conducted dilations initiated by endothelial agonists and to locate Cx37 into the plasma membrane. These functions are unaltered by long-lasting hypertension. In the presence of a nonconducting Cx40, Cx37 is present but cannot support the conduction highlighting the importance of endothelial Cx40.

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Key Words: conducted response ▪ gap junction ▪ microcirculation ▪ connexin ▪ hypertension

Arterioles in resting skeletal muscle exhibit considerable vascular tone to enable large increases in blood flow during exercise. However, large perfusion increases are only achieved if not only resistance vessels within the tissue but also upstream vessels dilate. Such an ascending dilation requires coordination within the vascular network. In addition to flow-induced dilation, the conduction of locally initiated dilatory signals along the vascular tree through intercellular channels contribute to the concerted activity of vascular cells. These channels are composed of connexin (Cx) protein subunits, which assemble into a hemichannel that docks face to face to its counterpart in the plasma membrane of an adjacent cell. The channels are clustered in specific regions named gap junctions providing a low resistance pathway for electrotonic transmission of vasoconstrictor signals. The Cx family is composed of 20 members named according to their molecular weight of which 4 are expressed in vessels, Cx40 and Cx37 mainly in endothelial cells and Cx43 in smooth muscle cells, which may additionally express Cx45. Cx40 is important in arteriolar coordination because the conductions of locally initiated endothelium-dependent dilations (conducted responses) are impaired in mice ubiquitously deficient for Cx40. This attenuation is restricted to endothelium-dependent dilators (acetylcholine [ACh] and bradykinin [BK]) whereas conducted dilations initiated by adenosine remained unaffected suggesting that the endothelial cell layer itself is crucial to transmit the signal. Moreover, these mice are hypertensive, which is caused by the abrogation of the negative feedback of renal perfusion pressure on renin secretion that consequently leads to inappropriate high levels of circulating renin. Accordingly, mice that lack Cx40 specifically in renin-producing cells are likewise hypertensive, whereas...
endothelial deletion of Cx40 does not alter arterial pressure.\textsuperscript{13} Hence, studying conducted dilations in these animals allows to identify the exact reason of the impairment observed in ubiquitous Cx40-deficient mice, that is, endothelial lack of Cx40 or long-lasting hypertension. This is of particular interest because hypertension itself suggestedly impairs the conduction of hyperpolarization and dilation on ACh\textsuperscript{14,15} and reportedly affected Cx expression.\textsuperscript{15–18} Strikingly, Cx37 expression is reduced in aortic endothelium in Cx40-deficient hypertensive mice\textsuperscript{19} and also in hypertensive rats.\textsuperscript{20} Furthermore, Cx37 is undetectable by immunofluorescence in arterioles in these mice\textsuperscript{3} raising the possibility that hypertension downregulates Cx37 expression and this downregulation impairs conducted responses in conjunction with deleted Cx40. Alternatively, Cx40 may act as a conducting channel of hyperpolarization and dilation on ACh\textsuperscript{14,15} which led to the substitution of alanine by serine at position 96 (Cx40A96S).\textsuperscript{21} This mutated Cx40 forms gap junction channels that are nonconducting and may be used to identify the role of Cx40 versus Cx37 in conducted dilations. We hypothesized that endothelial Cx40 is crucially important for Cx37 expression and conducted responses and that its deletion or mutation disrupts the conduction of endothelium-dependent dilations. To this end, we studied conducted responses and Cx37 expression in mice lacking Cx40 in endothelial or renin-producing cells as well as in mice expressing Cx40A96S.

Materials and Methods
Experiments were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals and approved by the local government. Mice carrying a floxed Cx40 gene (Cx40fl)\textsuperscript{13} were mated with animals carrying a Cre recombinase gene controlled by the Tie2 promoter or the renin promoter to generate Cx40\textsuperscript{fl} animals harboring Tie2-Cre (Cx40\textsuperscript{fl}:Tie2-Cre), Ren-Cre (Cx40\textsuperscript{fl}:Ren-Cre), or no Cre recombinase (Controls: Cx40\textsuperscript{fl}). A second group of animals carried a floxed and deleted (Cx40\textsuperscript{fl}) or a wild-type Cx40 allele (Cx40\textsuperscript{wt}) in conjunction with Tie2-Cre. Moreover, mice expressing the Cx40 mutation (Cx40A96S) were studied.\textsuperscript{21} Animals were anesthetized using medetomidin, fentanyl, and midazolam and prepared for intravital microscopy in the cremaster muscle.\textsuperscript{21} Arterioles were briefly stimulated using glass micropipettes containing vasoactive substances and responses studied locally and at upstream sites. Arterial pressure was measured by radioterym.\textsuperscript{13} Expression of Cx37 and Cx40 was analyzed by immunostaining\textsuperscript{6,8} and mRNA of Cxs by real-time polymerase chain reaction. Values are given as mean±SEM and compared within genotypes of the first experimental group a similar transient dilation was not delayed at 0.6 and 1.2 seconds. The peak dilation amounted to 61±3% (Cx40\textsuperscript{wt}), 66±3% (Cx40\textsuperscript{fl}:Tie2-Cre), and 59±2% (Cx40\textsuperscript{fl}:Ren-Cre; P=not significant between genotypes) and was achieved within 5 to 7 seconds. This dilution conducted rapidly to remote upstream sites, that is, peak dilation was not delayed at 0.6 and 1.2 mm in all genotypes. In fact, there was a trend toward a shorter time to peak at 1.2 mm in Cx40\textsuperscript{wt} (5.5±0.7 seconds, P=0.06 versus local) and time to peak was shorter in Cx40\textsuperscript{fl}:Tie2-Cre (5.6±0.5 seconds, P<0.01 versus local). The maximal amplitude of the dilation

Results
Experimental Groups and Resting Arteriolar Tone
The first experimental group consisted of animals deficient for Cx40 in endothelial (Cx40\textsuperscript{fl}:Tie2-Cre) or renin-producing cells (Cx40\textsuperscript{fl}:Ren-Cre) and controls (Cx40\textsuperscript{wt}). The maximal diameter of the arterioles studied ranged from 21 to 53 µm. Neither maximal (36±1 µm) nor resting diameters (12±1 µm) or vascular tone (0.34±0.02) were different between genotypes (Table S1, available in the online-only Data Supplement). A second experimental group consisted of animals carrying a deleted Cx40 allele, the floxed Cx40 allele, and Tie2-Cre (Cx40\textsuperscript{fl}:Tie2-Cre). Mice without Tie2-Cre (Cx40\textsuperscript{wt}) or the wild-type instead of the deleted Cx40 allele (Cx40\textsuperscript{wt}:Tie2-Cre) served as controls. Arteriolar diameters were also similar between genotypes in this and in the third group consisting of animals with the mutated Cx40 (Cx40\textsuperscript{A96S/A96S}) and wild-type littermates (Table S1).

Connexin Expression in Endothelial Cells
Immunofluorescence in Cx40\textsuperscript{wt} mice revealed dense expression of Cx40 in endothelial cells, located at cell borders in small arterioles of the cremaster muscle (Figure 1, lower resolution: Figure S1) and in the aorta (Figure S2). A similar quantity and pattern of Cx40 expression was found in Cx40\textsuperscript{fl}:Ren-Cre and expectedly Cx40 staining was lacking in Cx40\textsuperscript{fl}:Tie2-Cre mice. This verifies successful deletion of Cx40 in endothelial cells. The specificity was ensured by detection of Cx40 in atrial cardiomyocytes in all genotypes (Figure S3). The mutated Cx40 is located in the plasma membrane of endothelial cells because staining was indistinguishable between Cx40\textsuperscript{A96S/A96S} and wild-type mice (Figures 1 and S1).

Cx37 expression is altered in ubiquitous Cx40-deficient animals\textsuperscript{8,19} and was, therefore, assessed. Cx37 staining was intense at endothelial cell borders in the aorta (Figure S2) and arterioles of different sizes in the cremaster muscle (Figures 1 and S1) in Cx40\textsuperscript{wt} and Cx40\textsuperscript{fl}:Ren-Cre mice. However, Cx37 was never detected in endothelial cells in Cx40\textsuperscript{fl}:Tie2-Cre arterioles (Figure 1). In aortic endothelial cells, Cx37 was found at cell borders but staining was less bright in Cx40\textsuperscript{fl}:Tie2-Cre compared with other genotypes (Figure S2). Interestingly, Cx37 expression remained unchanged in arterioles and aorta of Cx40\textsuperscript{A96S/A96S} mice compared with wild types (Figure 1). In contrast to Cx40, Cx37 staining was not observed in cardiac atrial cells excluding antibody cross-reactivity (Figure S3). Transcriptional activity of Cx37, assessed by real-time polymerase chain reaction, was not altered in the cremaster muscle and the aorta of Cx40\textsuperscript{fl}:Tie2-Cre compared with Cx40\textsuperscript{wt}. Likewise, Cx43 and Cx45 mRNA expression was similar (Figure S4).

Attenuation of Conducted Responses Initiated by ACh and Bk in Mice With Defective Cx40
Brief, local arteriolar stimulation with ACh evoked in all genotypes of the first experimental group a similar transient dilation at the stimulation site starting at 1 second after stimulation and lasting for ≈20 seconds. The peak dilation amounted to 61±3% (Cx40\textsuperscript{wt}), 66±3% (Cx40\textsuperscript{fl}:Tie2-Cre), and 59±2% (Cx40\textsuperscript{fl}:Ren-Cre; P=not significant between genotypes) and was achieved within 5 to 7 seconds. This dilution conducted rapidly to remote upstream sites, that is, peak dilation was not delayed at 0.6 and 1.2 mm in all genotypes. In fact, there was a trend toward a shorter time to peak at 1.2 mm in Cx40\textsuperscript{wt} (5.5±0.7 seconds, P=0.06 versus local) and time to peak was shorter in Cx40\textsuperscript{fl}:Tie2-Cre (5.6±0.5 seconds, P<0.01 versus local). The maximal amplitude of the dilation
at distant sites was not attenuated in Cx40fl/fl (0.6 mm: 56±5%, 1.2 mm: 56±5%; both P<0.001 versus local). Figure 2A through 2C depicts dilations over time. In the second experimental group composed of animals being heterozygous for Cx40fl consistent results were obtained. The amplitude of the ACh dilation decreased with distance in Cx40fl:Ren-Cre mice (0.6 mm: 55±5%, 1.2 mm: 55±8%; both P=0.18 and P=0.37 versus local). In Cx40A96S/A96S animals, peak amplitude of the ACh dilation also decreased during conduction from 72±6% (local) to 49±7% (at 1.2 mm, P<0.001 versus local) but not in wild-type littermates (local 65±6%, 1.2 mm 64±5%; P=0.18 and P=0.37 versus local). The temporal behavior is depicted in Figure 3D through 3F. The decrease in peak amplitude at remote sites in Cx40A96S/A96S (0.6 mm −21±4%, 1.2 mm −23±9%) was not different from animals with endothelial Cx40 deletion (Cx40fl:Tie2-Cre: 0.6 mm −25±3%, 1.2 mm −28±2%, both P>0.47). In the second and third experimental groups, the occurrence of the peak amplitude was also not delayed at remote sites in either genotype. Response durations were not different between genotypes but tended to be shorter at further distant sites in all genotypes.

Bk, a distinct endothelium-dependent dilator, evoked likewise a transient, local dilation. The temporal characteristics of the local dilation differed from ACh such that it started later (≈3 seconds), reached its peak only within 24±1 seconds, and lasted longer (59±2 seconds). However, the dilation conducted to upstream sites without delay but the duration was shortened by ≈12 seconds in all genotypes of the first group. Markedly, the peak dilatory amplitude decreased during conduction only in Cx40fl:Tie2-Cre mice but not in controls (Figure 3A). Similar results were obtained in the second experimental group, that is, an attenuation of the amplitude at 1.2 mm in Cx40fl:Tie2-Cre (Figure 3B) without alteration of the temporal behavior. The peak amplitude was also reduced in Cx40A96S/A96S mice (group 3) leading to a significant difference of the dilatory amplitude at 1.2 mm compared with wild type despite similar local responses (Figure 3C). This decrease in peak amplitude in Cx40A96S/A96S (−21±9%) was not different from Cx40fl:Tie2-Cre animals (−31±5%, P=0.34).

Conduction of Dilations Initiated by Adenosine Is Unaltered by Endothelial Cx40 Deficiency

Application of adenosine elicited a local dilation peaking at ≈12 seconds and lasting for ≈35 seconds. It conducted without significant delay upstream but lasted shorter at remote sites (by ≈10 seconds). The peak dilatory amplitude decreased with distance in all genotypes of group 1 (Figure 3D) without differences between Cx40fl:Tie2-Cre and control mice. The amplitude at remote sites in hypertensive Cx40fl:Ren-Cre mice was significantly smaller compared with the other genotypes despite a similar local dilation. In all genotypes of experimental groups 2 and 3, the dilatory amplitude decreased similarly (Figure 3E and 3F) and also the temporal behavior was indistinguishable.
Local Application of High K+ Solution Induced a Biphasic Conducted Response

The confined application of 3 mol/L of K+ induced a biphasic response, an initial strong constriction lasting 5 seconds followed by a dilation with a smaller amplitude and a return to baseline within ~15 seconds (Figure 4A and 4E). The initial constriction peaked 2.1±0.1 seconds after application and was conducted to remote sites but the amplitude declined rapidly with distance (Figure 4B and 4F). The local constriction and its conduction was virtually similar in all genotypes (Figure 4D) including Cx40A96S/A96S (Figure 4H). In contrast, the secondary dilation conducted for distances up to 1.2-mm without amplitude decrease in animals expressing intact Cx40 (Cx40fl/fl, n=6) compared with wild types (wt; n=7). *P<0.05 and **P<0.001 peak amplitude vs local site (paired t test).

Arterial Pressure in Conscious Mice

Blood pressure was measured using telemetry. A circadian rhythm was evident 5 days after implantation as judged by higher pressures of ≈8 mm Hg in all genotypes during nighttime when mice are more active. Systolic, mean, and diastolic pressures were significantly elevated in Cx40A96S/A96S (by ≈25 mm Hg) compared with heterozygous and wild-type mice during day and night (Figure S5). However, heart rate was not elevated (day 497±28 versus 465±31, night 535±27 versus 525±22 per minute; wild type and Cx40A96S/A96S, respectively) and also pulse pressure amplitude was not different because systolic and diastolic pressures were increased by a similar amount. Importantly, pressure in heterozygous animals was not different from wild type.

Discussion

In the present study, we provide evidence that endothelial Cx40 is crucial for conduction of locally initiated endothelium-dependent dilations along the vessel wall. Loss of Cx40 in endothelial cells but not its lack in renin-producing cells, which is associated with hypertension, attenuated the amplitude of such dilations at remote sites. The lack of Cx40 in endothelial cells was associated with a decrease of Cx37 expression in arteriolar endothelium below the detection limit of immunofluorescence. However, the reduction of Cx37 expression was not essential for an impaired conducted dilation, because animals expressing Cx40A96S, which forms nonconducting channels, exhibited a similar decrease of the dilatory amplitude at remote sites, yet the expression of Cx37 was not affected in these animals. In addition, hypertension, which was verified in mice expressing Cx40A96S, is not causal for the decreased Cx37 expression in arteriolar endothelium, a finding that is
underlined by unaltered Cx37 expression in hypertensive mice devoid of Cx40 in renin-producing cells. Taken together, the present study demonstrates that a functionally conducting Cx40 is crucial to support unimpaired conduction of endothelium-dependent dilations along the vessel. Moreover, we suggest that Cx40 is required to locate or maintain Cx37 in the plasma membrane of endothelial cells. This latter function is independent of the conduction of a locally initiated constriction on high K+ solution was independent of Cx40 and the amplitude of conducted dilations initiated by adenosine was reduced in mice deficient for Cx40 in endothelial cells lacking Cx40 in renin-producing cells. This suggests that a conduction deficit per se does not cause hypertension and, conversely, hypertension does not affect the endothelial conduction pathway. The attenuation was specific for endothelium-dependent agonists (ACh and Bk), whereas the amplitude of conducted dilations initiated by adenosine was not affected. This is in line with our previous observations and the conclusion that dilations in response to adenosine conduct along a distinct pathway independent of endothelial Cx40. This pathway may involve the smooth muscle layer and is less efficient, which is reflected by the attenuation of the amplitude with distance as found also before. Herein, we demonstrate that the amplitude of conducted endothelium-dependent dilations is reduced in mice deficient for Cx40 in endothelial cells despite normotension but not affected in hypertensive mice lacking Cx40 in renin-producing cells. This suggests that a conduction deficit per se does not cause hypertension and, conversely, hypertension does not affect the endothelial conduction pathway.

Figure 3. Peak amplitude of dilations initiated by bradykinin (A through C) or adenosine (D through F) depicted as a function of distance from the stimulation site in 3 experimental groups. The first group (A and D) consisted of connexin40 (Cx40)fl/fl:Tie2-Cre and Cx40fl/fl:Ren-Cre mice with Cx40fl/fl controls. In (B and E) Cx40fl/fl:Tie2-Cre and respective controls (Cx40fl/wt and Cx40fl/wt:Tie2-Cre) are depicted (group 2). Group 3 animals (C and F) express the mutated (Cx40A96S) or wild-type (wt) Cx40. Peak dilations on bradykinin were attenuated upstream only in animals devoid of endothelial Cx40 or carrying the mutated Cx40. In contrast, the peak amplitude of dilations initiated by adenosine decreased with distance in all genotypes and controls in a similar manner apart from Cx40fl/fl:Ren-Cre, in which the decrease was enhanced. *P<0.05, **P<0.01, ***P<0.001 vs local (paired t test), #P<0.05 vs other genotypes (ANOVA), n=5 to 9 arterioles in ≥5 experiments in each group.
activation of the inward-rectifier K⁺ channel and the Na⁺/K⁺-ATPase in endothelial and smooth muscle cells at lower K⁺ concentrations resulting in a hyperpolarization.⁶⁻²⁹ The conduction of the dilation depended partially on endothelial Cx40 and resembled the ACh and Bk responses because the amplitude did not decrease for distances up to 1.2-mm in controls. This is consistent with the observation that destruction of the endothelial layer using a light-dye approach abolished the conduction of the secondary dilation in the hamster cheek pouch.²⁸ Thus, we conclude that the endothelial conduction pathway, which is highly efficient in transmitting dilatory signals, requires endothelial Cx40 but remains unaffected by hypertension. It is activated by ACh and Bk but also transmitting the secondary dilation in response to K⁺ stimulation. In the absence of Cx40, the efficacy of conduction resembles adenosine dilations indicating that the remaining dilatory signals are transmitted along the smooth muscle layer during these conditions.⁸

Surprisingly, Cx37 expression was not detected in arteriolar endothelium and reduced in aortic endothelium in endothelial Cx40-deficient animals as judged by immunostaining. The decrease in Cx37 protein was not because of an impaired transcription because mRNA levels were not reduced in these mice. The attenuation of Cx37 protein amount was previously observed in hypertensive mice being ubiquitously deficient for Cx40.⁸,¹⁹ The present study demonstrates that the strong reduction or possible abrogation of Cx37 expression is not related to hypertension because Cx37 was preserved in hypertensive animals (Cx40fl/fl:Ren-Cre) but indeed caused by the lack of Cx40 in endothelial cells. This important observation raises the question of whether the attenuation of the conduction described above is only found if both Cxs, Cx40 and Cx37, are lacking in arteriolar endothelium. Interestingly, Cx37 was located in endothelial cell membranes in animals expressing Cx40A96S, which forms nonconducting gap junction channels.²¹ However, despite the presence of Cx37 the
conduction of endothelium-dependent dilations was attenuated to a similar degree in these animals. This demonstrates first that Cx40 forming functional channels is required to sustain conducted dilations along endothelial cells and second that Cx40 is required in arterioles to locate Cx37 in the membrane. Interestingly, this latter function is maintained even by the mutated Cx40 and occurs despite hypertension. Immunostaining does not allow us to verify whether Cx37 proteins do indeed form functional channels in the membrane in these mice; however, in cell expression systems Cx37 alone is able to interconnect adjacent cells substantiating that Cx37 alone is capable of forming functional gap junction channels.

Animals homozygous for Cx40A96S were hypertensive as reported previously using tail-cuff plethysmography.22 We extended these findings by demonstrating elevated systolic and diastolic pressures in these mice during day and night. Despite hypertension, the animals exhibited a circadian and diastolic pressures in these mice during day and night. Most interestingly, heterozygous animals remained normotensive suggesting that defective Cx40 does not exert a rhythmic function of wild-type Cx40 in pressure regulation.

Perspectives

Cx40 serves in diverse cells as a crucial protein to allow intercellular signaling. Depending on the cell type, the physiological consequences are distinct and can be undoubtly dissected by cell-specific deletion, a powerful approach in animal research. However, Cx40 functions exceed beyond providing a gap junction pore because it recruits Cx37 to the endothelial cell membrane. This function is independent of conductivity and may be called scaffolding. Thus, the multiplicity of Cx expression in single cell types does not grant redundancy at the functional level which is highly relevant, considering their crucial functions in vascular and renal physiology. The close interaction of different Cxs in the endothelial plasma membrane may also prevail in other cells as suggested by the hypertension observed in animals expressing Cx40A96S. Unraveling the underlying mechanisms may provide further insight into gap junction physiology in the cardiovascular system as well as other organ systems.

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References


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**Novelty and Significance**

**What Is New?**

- Functional Cx40 is required in endothelial cells to support conduction of dilations initiated by endothelium-dependent agonists along the vascular wall.
- Cx40 is essential for the integration of Cx37 into the endothelial plasma membrane; however, Cx40 conductivity is not required for this function.
- Long-lasting hypertension itself does neither affect the conduction of dilations along the endothelial cell layer nor the expression of Cx37 in endothelial cells.

**What Is Relevant?**

- Gap junctions couple vascular cells and synchronize their behavior and endothelial cells provide a highly efficient signaling pathway along the vessel wall.
- Cx40 is the most important gap junction protein in endothelial cells and is also decisive in blood pressure control because of a function in renin-producing cells.

**Summary**

- A vascular conduction deficit per se does not cause hypertension and, conversely, hypertension does not affect the endothelial conduction pathway.

- The present study dissects Cx40 functions in different cells, that is, a conduction deficit caused by endothelial Cx40 deficiency can be separated from the hypertension caused by Cx40 deficiency in renin-producing cells. Despite the existence of a second endothelial connexin (Cx37), Cx40 takes center stage in part related to a newly identified close interaction of connexins at the cellular level. The presence of Cx37 with a nonconducting Cx40 does not preserve conducted responses, whereas hypertension itself does not impair the conducting capability of the endothelium.
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DEFECTIVE CX40 MAINTAINS CX37 EXPRESSION BUT INTACT CX40 IS CRUCIAL FOR CONDUCTED DILATIONS IRRESPECTIVE OF HYPERTENSION

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Materials and Methods

Mice

We mated mice that carry a floxed Cx40 gene with animals expressing a Cre-recombinase gene under the control of the TIE2 promoter (TIE2-Cre, obtained from M. Yanagisawa, University of Texas, Dallas, TX, USA) or the renin promoter (Ren-Cre, obtained from A. Gomez, University of Virginia, Charlottesville, VA, USA) to generate animals being homozygous for the floxed Cx40 gene and harboring either TIE2-Cre (Cx40fl/fl:TIE2-Cre), Ren-Cre (Cx40fl/fl:Ren-Cre), or no Cre-recombinase (controls, Cx40fl/wt). Thus, we created animals being deficient for Cx40 specifically either in endothelial cells or renin-producing cells. A second group of animals carried a floxed and deleted (Cx40fl/-) or a wildtype Cx40 allele (Cx40wt) in conjunction with TIE2-Cre providing a second line of endothelial Cx40-deficient mice. Moreover, mice expressing a Cx40 mutation (amino acid exchange at position 96 from alanin to serine, Cx40A96S) were studied and their wildtype littermates used as controls. This Cx40 mutation was identified in humans and these connexins form gap junction channels that are non-conducting. Mice were genotyped by PCR from tail-tip biopsies as described.

Experimental setup

Anesthetized male mice (medetomidin [0.5 mg/kg], fentanyl [0.05 mg/kg], midazolam [5 mg/kg] intraperitoneal) were prepared for intravital microscopy in the cremaster muscle. Animals were placed on a microscope stage with a heating pad to keep esophageal temperature at 37°C. Ventilation was facilitated by intubation through a tracheotomy and a catheter was placed in right jugular vein to maintain anesthesia by continuous infusion of the narcotic drugs. The right cremaster muscle was prepared for intravital microscopy and superfused with tempered (34 °C) saline solution (in mmol/L: Na+ 143, K+ 5, Ca2+ 2.5, Mg2+ 1.2, Cl− 127, HCO3− 25, SO42− 1.2 and H2PO4− 1.2) gassed with 5% CO2 and 95% N2 (pCO2 ≈ 40 mmHg, pO2 ≈ 30 mmHg) at a rate of 8 ml/min. After surgery the animal was placed onto the microscope (Axioskop FS, Carl Zeiss AG, Göttingen, Germany) equipped with a charge-coupled video camera (XC-77CE, PCO Computer Optics, Kelheim, Germany). Microscopic images were displayed on a monitor at 700-fold magnification and recorded on videotape for later measurement of luminal diameter after digitization.

Experimental Protocol

After 30 minutes of equilibration a glass micropipette filled with a vasoactive substances (acetylcholine [ACh], bradykinin [Bk], or adenosine [Ado]; 1 mmol/L) or depolarizing KCl solution (3 mol/L) was positioned in close proximity to a second- or third-order arteriole. A short pressure pulse (150 kPa, 100 to 500 ms) applied by a pneumatic ejector (PDES 02D, npi, Germany) onto the micropipette led to a locally confined stimulation of the arteriole. If a response at the site of stimulation (local) was observed, the microscope was moved to upstream sites at a distance of 0.6 mm or 1.2 mm and the stimulation was repeated. Responses at each site were always determined in duplicate before the maximal luminal diameter was measured for each site in the presence of a maximally dilating stimulus (combined superfusion of sodium nitroprusside, acetylcholine, and adenosine; each 30 µmol/L). Thereafter, vessels were allowed to reattain their resting diameters for 30 minutes before another vasoactive substance or a different arteriole was studied in the same manner. Experiments typically lasted 2 to 4 hours before the animal was killed by an intravenous injection of pentobarbital.

Measurement of arterial pressure by radiotelemetry

Only male mice were studied. Mice were anesthetized using isoflurane (1.5%) and surgically implanted with microminiaturized radiotelemeters (TA11PA-C10, Data Sciences International, St. Paul, MN, USA). The catheter was introduced into the left carotid artery and
advanced to the aortic arch as described. After stabilization and recovery (24 h) arterial pressure was recorded every five minutes for two minutes during five consecutive days at a 12 h light-dark cycle at 500 Hz.

**Immunohistochemistry**

The expression of Cx37 and Cx40 was analysed as well as the specificity of the cell type-specific deletion of Cx40 verified by immunostaining as described. Both cremaster muscles of an anesthesized mouse were prepared as described above and removed. Thereafter, the animal was killed using pentobarbital, two samples of the cardiac atrium were taken, the aorta removed, cut in two pieces, and opened lengthwise. All probes were pinned with their luminal site up flat in a dish covered with Sylgard®. After fixation (4.0% formaldehyde in phosphate-buffered saline [PBS], 5 min) and washing (PBS) preparations were blocked (2% BSA, 0.2% TritonX-100 in PBS, 2 h). Each tissue pair from a single animal was then incubated overnight at 4°C with a primary antibody (1:400 in blocking solution) targeted against Cx37 (CX37A11-A, Alpha Diagnostics, USA) or Cx40 (AB1726, Millipore, USA). After repetitive washing the tissues were incubated for 1 h at room temperature with a secondary antibody (Alexa Fluo 594, 1:800) and a nuleic acid stain (Sytox Green®, Invitrogen, USA). After intensive washing tissues were embedded using Mowiol® and staining was visualized on the following day by confocal laser scanning microscopy using appropriate wavelengths and filters (Leica, TCS SP5, Wetzlar, Germany).

**Real-time PCR**

Cx40fl/fl:TIE2-Cre and Cx40fl/fl controls were killed by intraperitoneal injection of pentobarbital (1 g/kg; Merial, Germany) and cremaster muscles as well as the aorta quickly removed. Organs were initially stored in RNAlater (Qiagen GmbH, Hilden, Germany) until further treatment. Proteins were digested using proteinase K overnight at 55°C before tissue disruption and homogenisation on the next day using a pestle and QIAshredder spin columns (Qiagen GmbH, Hilden, Germany). Tissue lysates of two mice were pooled and total RNA isolated using the RNeasy® Protect Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. Genomic DNA contamination was eliminated by digestion of the eluate using DNase1 (Fermentas GmbH, St. Leon-Rot, Germany). RNA concentration and purity was measured by means of a Nanodrop (ND-1000 UV/VIS, Peqlab, Erlangen, Germany). 450 ng of total RNA were transcribed into cDNA using SuperScript® III Reverse Transkriptase (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer’s protocol using Oligo(dT)22 primer (Eurofins MWG Operon, Ebersberg, Germany). A SYBR green based real-time PCR (SensiMix TM SYBR Hi-ROX Kit, Peqlab, Erlangen, Germany) was used for mRNA quantification of Cx37, Cx43 and Cx45. Gapdh (glyceraldehyde-3-phosphate dehydrogenase), Gusb (glucuronidase beta), and Rpl28 (60S ribosomal protein L28) mRNA were also determined and used for normalisation. Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany) with the following sequences: Cx37-forward 5´-TCC TGG GAA AAA GCA CTG AT-3´, Cx37-reverse 5´-CTG TGT CTG TCC AGG TGA CG-3´, Cx43 forward 5´-TCC TTT GAC TTC AGC CTC CA-3´, Cx43 reverse 5´-CCA TGT CTG GGC ACC TCT-3´, Cx45 forward 5´-ACA GGA GTT CTG GTG AAC AGG-3´, Cx45 reverse 5´-CTA GCA GGC GAG TCA GGA AG-3´, Gapdh forward 5´-TCA CCA CCA TGG AGA AGG C-3´, Gapdh reverse 5´-GCT AAG CAG CAG TTG GTG GTG CA-3´, Gusb forward: 5´-ACT GAC ACC TCC ATG TAT CCC AAG-3´, Gusb reverse 5´-CAG TAG GTC ACC AGC CCG ATG-3´, Rpl28 forward 5´-CAG ATC CGG TCA GCG AAA AC-3´, Rpl28 reverse 5´-GAT GCT GCT GAG GTG AGC CC-3´. 2 µl of the cDNA solution were utilized in each sample for amplification (50°C 2 min, 95°C 10 min, followed by 40 cycles of 95°C 15 sec and 60°C 1 min). Every gene was measured in triplicate in both tissues and mice. Threshold was set automatically by the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Applera, Darmstadt, Germany). The mean Ct value of
Gapdh, Gusb, and Rpl28 was calculated for each tissue and used to normalise Ct value of Cx and thus Cx mRNA levels.

**Statistics and calculations**

Duplicate measurements in one vessel are averaged and considered as single observation. Arteriolar diameter changes are normalized to the respective maximal possible response:

\[
\% \text{ of maximal response} = \frac{(D_{\text{stim}} - D_{\text{Co}})}{(D_{\text{Max}} - D_{\text{Co}})} \times 100
\]

where \(D_{\text{stim}}\) is diameter after stimulation, \(D_{\text{Co}}\) diameter before stimulation and \(D_{\text{Max}}\) the respective maximal diameter for dilation and minimal for constriction. Arterial pressure and heart rate values were averaged for day- and nighttime. Values are given as means ± SEM. Comparisons within groups were performed using paired t-test, data between groups using analysis of variance followed by Bonferroni correction for multiple comparisons. Significance was considered at \(P<0.05\).

**References**


### Table S1: Vascular diameters of the arterioles studied in three experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>genotype</th>
<th>animals</th>
<th>arterioles</th>
<th>resting</th>
<th>maximal</th>
<th>tone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cx40fl/fl:TIE2-Cre</td>
<td>10</td>
<td>36</td>
<td>12±1</td>
<td>37±1</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>1</td>
<td>Cx40fl/fl:Ren-Cre</td>
<td>8</td>
<td>31</td>
<td>12±1</td>
<td>37±1</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>1</td>
<td>Cx40fl/fl</td>
<td>11</td>
<td>38</td>
<td>13±1</td>
<td>34±1</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>2</td>
<td>Cx40fl-/-:TIE2-Cre</td>
<td>10</td>
<td>34</td>
<td>14±1</td>
<td>37±1</td>
<td>0.39±0.02</td>
</tr>
<tr>
<td>2</td>
<td>Cx40fl-/-</td>
<td>7</td>
<td>25</td>
<td>19±2</td>
<td>40±2</td>
<td>0.46±0.04</td>
</tr>
<tr>
<td>2</td>
<td>Cx40fl/fl:TIE2-Cre</td>
<td>8</td>
<td>26</td>
<td>19±2</td>
<td>37±1</td>
<td>0.49±0.04</td>
</tr>
<tr>
<td>3</td>
<td>Cx40A96S/A96S</td>
<td>7</td>
<td>25</td>
<td>13±1</td>
<td>33±1*</td>
<td>0.39±0.02</td>
</tr>
<tr>
<td>3</td>
<td>Cx40wt/wt</td>
<td>7</td>
<td>23</td>
<td>13±1</td>
<td>36±1</td>
<td>0.36±0.02</td>
</tr>
</tbody>
</table>

Data show number of animals and arterioles in the different experimental groups. Unstimulated (resting) and maximal diameter of the arterioles are given in µm. Tone is calculated as fraction of resting from maximal diameter. These values were not different within the three experimental groups between genotypes with the exception of maximal diameter in group 3 (ANOVA). *: P<0.05 vs. Cx40wt/wt.
Supplemental Figures

Figure S1

Lower resolution images of immunostaining for Cx40 (A-E, red) and Cx37 (F-J, red) in arterioles of the cremaster microcirculation in analogy to figure 1. Endothelial cell borders are brightly stained for Cx40 and Cx37 except for Cx40<sup>fl/fl</sup>:TIE2-Cre arterioles in which not only staining for Cx40 was lacking (B) but also staining for Cx37 (G). Scale bars are 50 µm, nuclei are stained using Sytox Green<sup>(R)</sup>.
Figure S2

Expression of Cx40 (A-E, red) and Cx37 (F-J, red) in aortic endothelial cells (en face view). In Cx40^fl/fl^ mice, endothelial cell borders in the thoracic aorta are brightly stained for Cx40 (A) and Cx37 (F) demonstrating abundant endothelial expression of both connexins. Animals deficient for Cx40 in endothelial cells (Cx40^fl/fl^:TIE2-Cre) did exactly not exhibit staining for Cx40 (B) verifying successful endothelial deletion of this connexin. Cx37 staining (G) was less bright as compared to controls indicating a reduction of Cx37 expression in the membrane. Connexin staining in aortas of hypertensive animals lacking Cx40 in renin-producing cells (Cx40^fl/fl^:Ren-Cre) was unchanged (C,H). The mutated Cx40 (Cx40A96S) was readily detectable (E) and located in the endothelial cell membranes in a fashion similar as wildtype Cx40 in littermates (D). Likewise, Cx37 expression in these mice (J) was unchanged from controls (I). Images are representative for 3 experiments in each genotype, scale bars are 20 µm, nuclei are stained using Sytox Green(R).
Expression of Cx40 (A-C, red) and Cx37 (D-F, red) in the atrium of the heart. Cx40 staining in atrial cardiomyocytes at cell borders was comparable in control (Cx40^{fl/fl}, A), endothelial-cell specific Cx40-deficient (Cx40^{fl/fl};TIE2-Cre, B) and renin-producing cell specific Cx40-deficient mice (Cx40^{fl/fl};Ren-Cre, C) indicating unchanged Cx40 expression in non-targeted cells. Expectedly Cx37 staining was not observed in cardiac cells which excluded antibody cross reactivity. Images are representative for 3 experiments in each genotype, scale bars are 20 µm, nuclei are stained using Sytox Green^{R}. 

Figure S3
Figure S4

Transcriptional activity of Cx37 (white), Cx43 (light grey) and Cx45 (dark grey) in the aorta and the cremaster muscle of Cx40fl/fl (plain) and Cx40fl/fl:TIE2-Cre mice (hatched). Ct values of connexins are normalized to the mean Ct value of Gapdh, Gusb and Rpl28 (delta Ct). Note, that Cx expression levels are substantially lower in cremaster tissue, which most likely reflects the reduced amount of vascular tissue in the overall sample. Values are given as mean ± SEM, n=6 experiments each genotype, significant differences were not found.
Arterial pressure was measured in the carotid artery using telemetry in conscious mice at day 5 after implantation continuously over 24 h in mice homozygous (Cx40^{A96S/A96S}, n=6) or heterozygous for Cx40A96S (Cx40^{wt/A96S}, n=4) and wildtype littermates (wt, n=6). Significantly higher blood pressure during night (P<0.05, paired t-test) indicates a circadian rhythm in all genotypes. Systolic (sys), mean, and diastolic (dia) pressure was elevated by a similar amount in Cx40^{A96S/A96S} during day and night compared to the other genotypes. Heterozygous animals were not different from wt.

* P<0.05, ** P<0.01, *** P<0.001 vs. other genotypes (ANOVA).