Impaired Conduction of Vasodilation Along Arterioles in Connexin40-Deficient Mice

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Abstract—Connexins have been hypothesized to play an important role in intercellular communication within the vascular wall and may provide a mechanistic explanation for conduction of vasomotor responses. To test this hypothesis, we studied the transmission of vasomotor responses in the intact skeletal muscle microcirculation of connexin40-deficient mice (Cx40−/−). Arterioles were locally stimulated with hyperpolarizing dilators (acetylcholine [ACh] as well as bradykinin [Bk]) or depolarizing K+ solution, and the resulting changes in diameter were measured using a videomicroscopy technique at the site of application and up to 1.32 mm upstream. Arterial pressure was elevated 25% in Cx40−/− mice (94±5 versus 75±4 mm Hg). Vessels selected for study had equivalent basal diameter and vasomotor tone in both genotypes of mice. Vasomotion was present in small arterioles of both genotypes, but its intensity was exaggerated in Cx40−/− mice. ACh and Bk induced dilation (33% and 53%, respectively, of maximal response) at the site of application that was of similar magnitude in both genotypes. These dilations were observed to spread upstream within <1 second without significant attenuation in Cx40−/− mice. However, spreading was severely attenuated in Cx40−/− animals (11±4% versus 35±7% with ACh and 38±5% versus 60±7% with Bk in Cx40−/− and Cx40+/+, respectively; P<0.05). In contrast, conducted vasoconstrictions, induced by K+ solution decreased equally with distance in both genotypes. These results support a significant role for Cx40 in vascular intercellular communication. Our observations indicate that Cx40 is required for normal transmission of endothelium-dependent vasodilator responses and may underlie altered vasomotion patterns. (Circ Res. 2000;86:649-655.)

Key Words: hypertension ■ acetylcholine ■ bradykinin ■ endothelium ■ gap junctions

The regulation of blood flow in a wide dynamic range requires coordinated responses of resistance and feeding arteries. Such a coordination between vessels can be achieved by the vascular effects of shear stress exerted by the streaming blood or by conduction of vasomotor signals along cells of the vascular wall. Indeed, local application of certain vasoactive compounds, such as acetylcholine (ACh) or nor-epinephrine (NE) induced not only local dilation or constriction but also vasomotor responses several millimeters upstream and downstream. Vasomotor responses can also be conducted from capillaries to arterioles and may contribute to the matching of tissue demands and blood supply. This has been demonstrated in the following way: When single muscle fibers were stimulated to contract, arterioles upstream of capillaries supplying these fibers were observed to dilate.

The high conduction velocity is consistent with electrotonic transmission of a signal along the vascular wall. In fact, locally induced hyperpolarizations and depolarizations have been demonstrated to be conducted several millimeters upstream in endothelial and vascular smooth muscle cells. The conduction of the electrical signal requires coupling of vascular cells by gap junctions that provide conduits of low electrical resistance between the cells. In vascular tissue, at least three different connexin (Cx) proteins (Cx37, Cx40, and Cx43) are expressed that form gap junctions. Cx40 seems to be the predominant connexin isoform in aortic endothelial cells, whereas in smooth muscle, Cx43 expression is abundant. Unfortunately, little information on the distribution pattern of connexins at the microcirculatory level is available.

In the present study, we investigated the functional role of Cx40-containing gap junctions in the conduction of vasomotor signals, using mice deficient in this connexin protein. Hyperpolarizing endothelium-dependent vasodilators, depolarizing K+ solutions, or NE was applied locally as a short pulse, and the resulting conduction of vasomotor responses was studied in wild-type and in Cx40-deficient mice. Given that we hypothesized that a potential defect in vascular cell coupling could alter peripheral vascular resistance resulting in hypertension, we also investigated arterial pressure in these animals.

Materials and Methods

Experimental Setup

Experiments were conducted in accordance with the German animal protection law. Cx40-deficient mice (Cx40−/−) and wild-type...
littermates (Cx40+/+), were anesthetized by droperidol (20 mg/kg), fentanyl (0.1 mg/kg), and midazolam (2 mg/kg IP), followed by continuous infusion (jugular vein). Arterial pressure was measured in the carotid artery, and heart rate was obtained from pressure pulse. The cremaster muscle was prepared as described15 and superfused the carotid artery, and heart rate was obtained from pressure pulse. The cremaster muscle was prepared as described15 and superfused the carotid artery, and heart rate was obtained from pressure pulse.

**Experimental Protocols**

After stabilization (30 minutes), sodium nitroprusside (SNP, 10 μmol/L) followed by ACh (10 μmol/L) was superfused. To study conduction of vascular responses, a micropipette (tip 1 to 2 μm) was positioned in close proximity to the arteriolar wall. ACh (10 mmol/L), bradykinin (1 mmol/L), NE (1 mmol/L), or KCl or NaCl (3 mol/L) was applied by pressure ejection (60 to 180 kPa, 100 to 600 ms) to obtain a response at the site of stimulation. The same stimulation was then used, and responses were studied at locations 0.33, 0.66, 0.99, and 1.32 mm upstream. Stimulations with KCl were repeated in the presence of phentolamine (0.1 μmol/L). Maximal diameter was measured during combined superfusion of adenosine, SNP, and papaverine (10 μmol/L each).

**Immunohistochemistry**

After anesthesia, the vasculature was flushed with saline through the left ventricle and the tissue was fixed with paraformaldehyde (2%). The cremaster muscle was embedded in OCT (Tissue Tek) and frozen in isopentane (−160°C). Sections (6 μm) were blocked with Tween (0.1%) and BSA (4%) and were immunolabeled with rabbit anti-Cx4016 and goat anti-factor VIII–related antigen (BIOTREND). Immunocomplexes were visualized using Cy2-conjugated and Cy3-conjugated donkey IgG (DIANOVA).

**Statistics and Calculations**

Vascular tone is expressed as a quotient of resting diameter divided by maximal diameter. Diameter changes were normalized to the maximal possible constriction or dilation: % of maximal response = (Dm − Do)/(Dm − Dco)×100, where Dm represents the diameter after treatment and Dco is the control diameter. Dco represents the maximal observed change in diameter (dilation or constriction). The “time to peak response” (interval between stimulus application and attainment of peak diameter) and the “response duration” (interval between stimulus and 50% of recovery) were calculated. The latter was used to calculate the integral of the dilation.

Comparisons within groups were performed using paired t tests and corrected according to Bonferroni. Data between groups were compared with ANOVA followed by post hoc analysis of the means or by Kruskal-Wallis test. Data are presented as mean±SEM. Differences were considered significant at a corrected error probability of P<0.05.

**Results**

**Arteriolar Resting Tone and Dilations Induced by Superfusion of Vasomotor Stimuli**

A total of 52 arterioles were studied in 28 mice. The mean maximal diameters of arterioles were not significantly different between genotypes (Cx40+/+: 39.3±2.3 μm; Cx40−/−: 37.9±2.1 μm). The vessels exhibited a varying degree of resting tone from 0.24 to 0.96 (mean: 0.63±0.03). In both genotypes, arteriolar resting tone was significantly higher in small (maximal diameter <35 μm) compared with large (maximal diameter >35 μm) vessels (Cx40+/+: small: 0.52±0.06, large: 0.74±0.04, n=10 and 16, P<0.05;
Cx40−/−: small: 0.50±0.07, large: 0.72±0.04, n=9 and 12, P<0.05). The addition of ACh (10 μmol/L) to the superfusion fluid induced nearly maximal dilation in Cx40−/− mice, whereas in Cx40−/− animals, it was attenuated (Figure 1). In contrast, dilations in response to the nitric oxide donor SNP were not different (Figure 1).

Local Stimulation With Endothelium-Dependent Dilators

Local stimulation of the arterioles in wild-type mice with a short pulse of ACh induced a dilation that reached within 3.4±0.5 seconds (time to peak) a maximum of 35.6±4.1% at the stimulation site and lasted for 21.0±5.0 seconds (response duration). Although maximal dilation (32.8±5.6%) and time to the peak response (3.3±0.4 seconds) were virtually identical at the local site in Cx40−/− mice, the response duration was significantly shortened (8.8±2.2 seconds, P<0.05 versus Cx40−/−). The local dilation was rapidly conducted upstream in both genotypes (Figure 2; the Table provides resting and maximal diameters). In wild-type mice, the maximal amplitude did not decrease up to 1.32 mm upstream (34.8±6.9%), and the time to peak value was not different (4.1±0.7 seconds). However, with increasing distance, the response became shorter (8.7±1.3 seconds, P<0.05 versus local site). Although the local dilation on ACh application was of virtually the same amplitude in Cx40−/− mice, upstream dilations were significantly attenuated compared with wild-type mice (Figure 2). The dilation 1.32 mm upstream was reduced to a maximum amplitude of 11.2±4.3% in Cx40−/− mice (P<0.05 versus local site). The response duration was only 3.5±1.3 seconds (P=0.07 versus local site) and, thus, like the local response, significantly shorter than the response in Cx40−/− mice. When ACh was applied at a distance of 0.2 mm from the vessel wall, no significant changes in diameter were found (15.0±1.4 versus 16.5±1.9 μm, n=6).

Bradykinin induced a local dilation of more than 50%, which was not different in both genotypes. As was found with ACh, the upstream dilation was not attenuated in wild-type mice, whereas it decreased in amplitude with increasing distance in Cx40−/− animals (Figure 3). Response durations were virtually identical in both genotypes at all different sites studied (Figure 3) as was the time to peak response (data not shown).

**Local Application of High K+ Solution**

Application of 3 mol/L K+ through the micropipette induced a rapid constriction, which was also conducted upstream. In contrast to endothelium-dependent dilators, there was a monotonic decrease of the conducted response with increasing distance in both genotypes (Figure 4). The maximal response decreased from −39.1±4.5% and −39.6±5.4% to −10.2±4.2% and −13.3±2.8% (Cx40+/+ and Cx40−/−, respectively). There were also no significant differences in time to peak response (data not shown), and duration of the response was not different at the local site (5.1±0.4 seconds in both genotypes). In contrast, duration of the responses was significantly longer in Cx40−/− at the most upstream sites (0.99 mm: 4.5±0.7 versus 2.3±0.7 seconds, P<0.05; 1.32 mm: 4.2±0.8 versus 1.9±0.7 seconds). Again, application of K+ solution 0.2 mm apart from an arteriole did not induce significant changes in diameter (36.8±2.0 versus 36.5±2.0 μm, n=9). If NaCl (3 mol/L) was applied instead of KCl, diameters remained unaffected at the site of application (38.7±3.7 versus 38.0±3.0 μm after 4 seconds, n=8) as well as at upstream sites (data not shown).
To study the role of NE release from perivascular nerves in the conduction process, vessels were stimulated with K\textsuperscript{+} solution before and after superfusion of the \(\alpha\)-receptor antagonist phentolamine (0.1 \(\mu\)mol/L). The efficacy of the blockade was verified by the abrogation of the constriction induced by superfusion of NE (0.1 \(\mu\)mol/L), which amounted to \(-23.8\pm11.1\%\) in the absence of the antagonist. However, phentolamine did not affect the constriction induced by K\textsuperscript{+} application at the local (\(-41.5\pm8.0\%\) versus \(-49.1\pm8.0\%\)) or upstream sites (0.3 mm: \(-29.1\pm4.6\%\) versus \(-33.7\pm3.7\%\); 0.66 mm: \(-13.1\pm2.4\%\) versus \(-16.6\pm3.3\%,\) before and after blockade of \(\alpha\)-receptors, respectively, \(n=7\)).

**Local Application of NE**

Local application of NE induced a strong constriction, which reached within 8.2\pm1.4 seconds a maximum of \(-43.2\pm4.3\%\) and lasted for 15.1\pm2.9 seconds (\(n=9\)). Thus, these constrictions were similar to those initiated by local KCl application (see Figure 4). Nevertheless, no significant constrictions were observed at upstream sites. The maximal constriction at the nearest observed upstream site (0.33 mm) amounted to \(-2.2\pm1.3\%\) \((P=0.09)\).

**Immunostaining**

To confirm the presence of Cx40 in wild-type mice in cremasteric vessels, immunohistochemistry was performed on cryosectioned tissue. Cx40 staining was revealed in cremasteric vessels (Figure 5B). The Cx40 antibodies colocalized with antibodies directed against the endothelial marker anti-factor VIII–related antigen (Figure 5C). Cx40 staining was not detected in cremaster vessels from Cx40\(^{-/-}\) mice (Figures 5D through 5F).

**Arterial Pressure**

The mean arterial pressure in wild-type mice (\(n=10\) animals) remained throughout the experiment between 71 and 76 mm Hg, which is in a range reported by others for anesthetized mice.\(^{17}\) In sharp contrast, the arterial pressure in Cx40\(^{-/-}\) animals (\(n=9\)) was significantly elevated by \(\approx25\%\) (Figure 6). Heart rate did not differ between both genotypes (Figure 6).

**Conducted Responses Induced by ACh in Other Hypertensive Mice**

The conduction of endothelium-dependent vasodilation was additionally studied in eNOS-deficient mice, which are also characteristically hypertensive.\(^{18}\) Arterial pressure was 99\pm6 mm Hg in our experiments. Application of ACh induced a dilation that amounted to 36.1\pm6.5\% 5 seconds...
after stimulation. The dilation was rapidly conducted upstream. Thus, 5 seconds after stimulation, a dilation of 34.9±4.7% at a distance of 0.66 mm and 30.1±3.5% at 1.32 mm distance was observed (n=7 vessels in 3 animals). These values were not significantly different from normotensive Cx40+/+ mice (local: 37.4±4.6%, 0.66 mm: 28.4±3.9%, and 1.33 mm: 29.2±7.0%).

Discussion

Our results show that Cx40 has a significant role in the propagation of vasodilations, initiated by local application of ACh or bradykinin. The propagation of vasodilations within the microcirculation in the mouse is likely due to vascular cell communication, which is substantially impaired in Cx40-deficient mice. Interestingly, the spread of vasoconstrictor responses initiated by a depolarizing K+ solution was not different between genotypes. Data were acquired in 10 (Cx40+/+) and 9 animals (Cx40−/−). *Significant differences.

Figure 6. Arterial pressure (top) in anesthetized wild-type mice (○) was in a range between 71 and 77 mm Hg over the complete experimental period. In contrast, the arterial pressure in Cx40-deficient animals (●) was elevated by ∼25%. Heart rate (bottom) was not different between genotypes. Data were acquired in 10 (Cx40+/+) and 9 animals (Cx40−/−). *Significant differences.

From data obtained in vitro, a length constant of 0.7 mm was calculated for the changes of membrane potential, whereas length constants for vasomotor responses were considerably higher (∼2 mm) but varied in different tissues. In the mouse cremasteric vessels, we did not find a decrease of the vasomotor response up to our maximal observation distance of 1.32 mm (Figures 3 and 4), which suggests that a regenerative mechanism might be involved. Therefore, we term the spreading of these endothelium-dependent dilations “propagation” to delimit these responses from passive conduction. The local release of EDHF leading to hyperpolarization, which might have contributed to the observed length constants, is less compromised during neuroleptanalgesia compared with pentobarbital narcosis, which was mostly used in hamsters. Additionally, dissipation of conducted vasomotor responses has been reported to be related to arteriolar branching structure, which might vary between species.

Local depolarization by elevation of the K+ concentration initiated upstream vasoconstrictions that were not due to the release of adrenergic neurotransmitters released from perivascular nerves because the effective blockade of α-adrenergic receptors did not affect the responses. Moreover, changes in osmolarity could not account for the observed phenomenon in view of the fact that similar application of NaCl, exhibiting identical osmolarity, did not induce significant changes in diameter. Thus, it is likely that smooth muscle depolarization initiated the responses. The incompetence of locally applied NE to induce conducted responses might relate to only minimal depolarization induced by this substance. It has to be kept in mind that endothelial depolarization might have contributed to the K+ -induced constriction, because endothelial cell depolarization, which should occur during K+ application, would decrease the driving force for endothelial Ca2+ influx, and release of endothelial vasodilators, as a result, decreases. However, because of the observed time course, depolarization of smooth muscle and subsequent constriction are most likely the underlying mechanisms. Interestingly, constrictions decayed with increasing distance in marked contrast to endothelium-dependent dilations. This divergence of length constants suggests that the changes in membrane potential propagate along different pathways, presumably endothelial and vascular smooth muscle cells. It is not clear to what extent both cell types are coupled by heterocellular gap junction channels. In vitro data support the notion that heterocellular coupling exists. In contrast, from measurements obtained in the hamster microcirculation in vivo, it has been proposed that each cell layer, i.e., the endothelium and the smooth muscle, forms a separate electrical pathway. From our experiments, conclusions about the pathways used cannot be readily deduced. However, our observation of different length constants for endothelium-dependent dilators and K+ solution would be consistent with separate electrical pathways.

Most importantly, upstream dilations in response to local application of endothelium-dependent dilators (ACh, bradykinin) were diminished in Cx40-deficient vessels. This attenuation is not related to the hypertension found in these animals because eNOS-deficient mice, which are character-
istically hypertensive,18 did not show such an attenuation. In marked contrast to endothelium-dependent dilations, the conduction of the depolarization-induced constrictions was not altered. This divergent effect of Cx40 channel expression can be explained in two ways. First, Cx40-containing gap junction channels may have a significant role in endothelial cell coupling but not in the communication of smooth muscle cells. Second, such gap junctions channels may be critical in heterocellular coupling of endothium and vascular smooth muscle. Heterocellular coupling has been described to play a central role in endothelium-dependent relaxations.31 The impairment of propagation of endothelium-dependent dilations fits with our observations on the expression of connexins in vascular tissue. We found coexpression of Cx40 and of the endothelial marker von Willebrand factor in cremasteric microvessels of wild-type mice (Figure 5), which indicates that mostly endothelial cells express this protein. Similarly, in large rat arteries (aorta), Cx40 is expressed most abundantly in endothelium and less frequently in smooth muscle cells.11,12 However, Cx40 and Cx43 were found in endothelial as well as vascular smooth muscle cells in the cheek pouch microcirculation of hamsters.13 The exact localization of these connexins in the microcirculation, which may help to decide between the proposed hypotheses, ie, homocellular and/or heterocellular coupling, remains unclear. Our data, however, demonstrate for the first time a functional role of Cx40 in the microcirculation, given that its absence severely impairs propagation of endothelium-dependent vasodilator signals along the arteriole. However, even in the absence of Cx40, a remaining dilation was found at upstream sites. This points to a role of gap junction channels formed by other connexins that mediate the remaining propagation along endothelial or smooth muscle cells or between these cell types.

Surprisingly, the Cx40-deficient mice were hypertensive as judged by anesthetized blood pressure values. In the absence of any modulation of the level of anesthesia attributable to the Cx40-deficient phenotype, this indicates a role of vascular Cx40 in the control of blood pressure. Currently, we do not know which mechanisms are involved. However, our data yield some clues. First, the endothelium-dependent dilation in response to ACh was impaired as derived from superfusion experiments, which may be explained by a reduced responsiveness to ACh in partially uncoupled cells. It has been shown in the lung microcirculation that part of the intracellular Ca2+ increase upon ACh stimulation was due to an influence of neighboring cells.34 Second, the lack of coordination between downstream and upstream vessels as demonstrated may increase peripheral resistance. Third, we observed in 3 of 10 Cx40-deficient mice, a spontaneous and quite irregular vasomotion that did not occur in wild-type animals. The arterioles constricted completely over a short length for several seconds, giving the impression of local spasms and leading to complete flow cessation. These contractions occurred repetitively and, if observed once, could be repeatedly identified along the vessel. These phenomena, which may occur also in other organs, could result in increased vascular resistance as well.

To our knowledge, the data in the present study provide the first experimental proof for a functional role of Cx40 in intercellular signaling underlying propagated vasodilation and in control of blood pressure. Most likely, other connexins also contribute to these effects. It will be challenging to dissect the interactions and complementary functions of different connexin proteins in the molecular physiology of the vessel wall using other targeted mouse mutants.

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References


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