Gap junctions regulate vessel diameter in chick chorioallantoic membrane vasculature by both tone-dependent and structural mechanisms

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Abstract
Objective: In this study, we examined the impact of gap junction blockade on chick chorioallantoic membrane microvessels.

Methods: Expression of Cx37, Cx40/42, and Cx43 in chick chorioallantoic membrane tissue was studied by PCR, Western blot, and confocal immunofluorescence microscopy. Vessel diameter changes occurring under gap junction blockade with carbenoxolone (175 µmol/L), palmitoleic acid (100 µmol/L), ⁴³GAP27 (1 mmol/L) were analyzed by intravital microscopy. To analyze vascular tone, chick chorioallantoic membrane vessels were exposed to a vasodilator cocktail consisting of acetylcholine (10 µmol/L), adenosine (100 µmol/L), papaverine (200 µmol/L), and sodium nitroprusside (10 µmol/L).

Results: In chick chorioallantoic membrane lysates, Western blot analysis revealed the expression of Cx40 and Cx43. Immunofluorescence in intact chick chorioallantoic membrane vasculature showed only Cx43, limited to arterial vessel walls. Upon gap junction blockade (3 hours) arterial and venous diameters decreased to 0.50 ± 0.03 and 0.36 ± 0.06 (carbenoxolone), 0.72 ± 0.08 and 0.63 ± 0.15 (palmitoleic acid) and 0.77 ± 0.004 and 0.58 ± 0.05 (GAP27), relative to initial values. Initially, diameter decrease was dominated by increasing vascular tone. After 6 hours, however, vessel tone was reduced, suggesting structural network remodeling.

Conclusions: Our findings suggest a major role for connexins in mediating acute and chronic diameter changes in developing vascular networks.

Abbreviations: Ach, acetylcholine; ADO, adenosine; ART, arteries; CAM, chick chorioallantoic membrane; CBX, carbenoxolone; CFM, confocal immunofluorescence microscopy; Cx, connexin; Cx37 (GJA4), connexin 37; Cx40 (GJA5), connexin 40; Cx43 (GJA1), connexin 43; DIP, diprydamole; GAP27, ⁴³GAP27; GJ, gap junction; HH, Hamburger Hamilton; LDH, lactate dehydrogenase; PA, palmitoleic acid; PAP, papaverine; PBS, physiological salt solution; RT-PCR, Reverse transcription polymerase chain reaction; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor; VEN, veins; VT, vascular tone.

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1 | INTRODUCTION

Gap junctions mediate intercellular communication. Composed of two hexamer transmembrane spanning hemichannels, called connexons, they allow the exchange of small molecules (<1 kDa) and ions by bridging the extracellular space of neighboring cells thereby connecting their cytoplasm. Each connexon consists of six Cx. Among the more than 20 Cx isoforms Cx37 (GJA4), Cx40 (GJA5), and Cx43 (GJA1) are the ones predominantly expressed in blood vessels.

Several studies have shown the importance of Cxs for vascular function and development. Knockout mice lacking GJA4 and GJA5 show severe vascular dysmorphogenesis with distended blood vessels and hemorrhages. In vitro, siRNA knockdown of GJA4, GJA5, or GJA1 in human umbilical vein endothelial cells resulted in decreased branch formation in tube formation assays. The group of Duling showed that knockout of endothelial Cx43 resulted in hypotension, bradycardia, and elevated plasma levels of NO and angiotensin I and II in mice. Furthermore, GJ-based communication is considered essential for the propagation of vasomotor responses along arterial vessels.

However, our current understanding of vascular GJs and their function in vascular maintenance and adaptation is mainly based on in vitro studies which can not address the interdependency and interactions of coupled blood vessels in microvascular networks of the living organism. Pioneering in vivo experiments analyzing the role of GJs in the murine microcirculation were carried out by the groups of Segal and DeWit. Both authors showed the importance of Cxs in mediating vasomotor responses and dissected the different conduction pathways involved. Still, even these groundbreaking experiments were limited to the analysis of single blood vessels and only suitable for investigating acute (but not prolonged) vessel changes. Consequently, the role of GJs for homeostasis and adaptation of functional microvascular networks remains unclear.

In previous work, we proposed a possible role for GJs-based communication in structural adaptation of vascular beds. Based on mathematical models for adaptation of vessel diameters, we predicted that microvascular networks regulated by local hemodynamic (pressure, shear stress) and metabolic stimuli cannot generate functionally adequate flow distributions unless information transfer along the wall of blood vessels (conducted response) also acts as a stimulus for structural adaptation. Conversely, absence of conducted stimuli is expected to lead to functional shunting and malperfusion.

To test this hypothesis, we investigated the role of vascular Cxs in the regulation of vessel diameters in functional vascular networks of the CAM model of fertilized chicken eggs. Since conducted responses may contribute to both acute (tone-dependent) and structural regulation of diameters, we also observed vessel diameters when exposed to a vasodilator cocktail. Changes in diameters of fully dilated vessels were considered to represent structural remodeling.

2 | MATERIALS AND METHODS

2.1 | Ex ovo CAM model

The ex ovo CAM model was used as previously described. Briefly, fertilized white leghorn chicken eggs (Gallus gallus, VALO BioMedia GmbH) were incubated in horizontal position at a temperature of 37.5°C and relative humidity of 60%-80%. After 72 hours, the eggshell was cracked under aseptic conditions; the embryonated eggs were placed in plastic culture dishes (TPP, internal diameter: 88 mm) and kept in a humidified environment at 37.5°C until chick embryos reached HH stage 40-42, which corresponds to day 14 of embryonic development.

2.2 | Intravital microscopy

For intravital microscopy, culture dishes were placed on a custom-made, temperature and humidity-controlled microscopic stage. The microscope (Axioskop) was equipped with two objectives (2.5×, NA 0.085; 5×, NA 0.16; Zeiss, transillumination) and CAM vascular networks were visualized for up to 24 hours. Microscopic images were taken using a CCD camera (C9900, MBF Bioscience) and processed by appropriate image analysis software (Neurolucida, Vs8; MBF Bioscience). Video sequences were recorded using a CMOS camera (Sony ICE600). Vessel diameters were measured using ImageJ Vs 1.51n software. After treatment with Cx blockers, a fraction of blood vessels could not be visualized anymore due to diameter reduction and impaired blood flow (see Figure 2A). Their post-treatment diameter was arbitrarily set to 5 μm.

2.3 | Reverse transcription polymerase chain reaction

Total RNA was extracted from CAM samples at development day 14 and purified using RNeasy Mini Kit following the manufacturer’s instructions (Qiagen). Samples were treated with ribonuclease-free deoxyribonuclease (DNASEI; Qiagen) to remove any genomic DNA contamination. RNA concentration and purity were measured spectrophotometrically at wavelengths of 260 and 280 nm, respectively. cDNA was synthesized by RT of 2 μg of total RNA using the Phusion RT-PCR Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Reverse transcription PCR was carried out in a total volume
of 50 µL containing 250 nmol/L of each primer, 500 µmol/L deoxyribo-
nucleotide triphosphate, 25 mmol/L MgCl₂, and 1 U Phusion Hot
Start II DNA-Polymerase. Primer pairs were combined and diluted
to a final concentration of 10 µmol/L. Primer sequences, annealing/ex-
tension temperatures, and gene bank accession numbers are listed in
Table 1. RT-PCR was performed in a Peltier thermal cycler (PTC-200;
MJ-Research).

2.4 | Western blotting

Western blotting was performed as described.²⁵ CAM tissue
lysates were prepared by excision of 5 mm² of CAM tissue, which
was washed two times with ice-cold PBS, lysed in ice-cold RIPA
buffer supplemented with protease inhibitors (Roche cOmplete,
Mini Protease Inhibitor Cocktail) and subsequently homogenized
with a homogenizer (Minitisli, Bertin Instruments). Homogenates
were centrifuged at 20 000 g, and supernatant was collected for
protein quantification analysis using BCA Thermo Scientific
Pierce™ Protein Assay. Fifteen-twenty microgram of protein was
loaded on an SDS-PAGE gel. Proteins were transferred to nitro-
cellulose membrane using a wet blot tank system (Bio-Rad) for
2 hours. Membranes were blocked for 1 hour at room temperature
with 5% skim milk in TBS-T before incubation with primary
(overnight, 4°C) and secondary (1 hour, room temperature) anti-
bodies in TBS-T. In between and after incubation, membranes were
washed three times with TBS-T at room temperature for 10 min-
utes. Antibody-stained proteins were visualized using the Fusion
SL system from Vilber and ECL Select Western Blotting Detection
Reagent (GE Healthcare).

2.5 | Antibodies

Given the high conservation of amino acid sequences of Cx iso-
forms in different species, we were able to use commercially avail-
able, rabbit polyclonal, primary antibodies directed against murine
(Cx37), or human Cxs (Cx40, Cx43) to test for the expression of
chick Cxs.²² GJA5 encodes for Cx42 in chicken and Cx40 in mam-
als and we used two different Cx40 antibodies to analyze its ex-
pression patterns. Table 2 shows the primary antibodies used for
immunoblotting and immunofluorescence and sequence identity
between their respective binding regions in mice/humans and chick
(as given by Basic Local Alignment Search Tool [BLAST] analysis,
available at https://www.ncbi.nlm.nih.gov). Selectivity and specific-
ity of the used antibodies were investigated by checking whether
Western blots revealed a single protein band at the expected mol-
ecular weight (Figure 1). Negative controls were performed by
omitting the primary antibody. For immunoblotting, secondary,
peroxidase-labeled antibodies (Vector Laboratories, PI-1000 and
PI-2000) were used at 1:3000-1:10 000. For immunofluorescence,
primary antibodies against desmin (Dako, M0760292) were used at
1:33. Secondary fluorescence-labeled antibodies (goat anti-mouse
568, A11031 and goat anti-rabbit 488; Invitrogen) were diluted in
a ratio of 1:200.
TABLE 2 Primary antibodies used for immunoblotting and immunofluorescence

| Name       | Antibody name (company) | Antibody-binding site                                      | Sequence identity between antibody-binding site and respective amino acid sequence in chick | Dilution for immunoblotting (immunofluorescence) |
|------------|-------------------------|------------------------------------------------------------|---------------------------------------------------------------------------------------------|--------------------------------------------------|
| Cx37 (Gja4) | Ab58918 (Abcam)         | N-terminal amino acids 73-122 of murine Cx37               | 80% (40/50)                                                                                 | 1:2000                                           |
| Cx40/42 (Gja5) | SAB1304973 (Sigma)     | N-terminal region of human Cx40                             | 89% (158/177)                                                                               | 1:1000 (1:200)                                  |
| Cx43 (Gja1)  | C6219 (Sigma)           | C-terminal amino acids 363-382 of human Cx43               | 95% (19/20)                                                                                 | 1:5000-1:10 000 (1:100)                         |

\(^a^\) The SAB1304973 antibody targets the N-terminal sequence of human Cx40. Here, sequence identity between chick and human is 89% (158/177). \(^b^\) The binding site was of the Ab183648 antibody was not specified. According to the manufacturer, species reactivity includes chick Cx 42.

2.6 Confocal immunofluorescence microscopy

For imaging and staining of vascular membranes, 5 mm² of CAM tissue was excised from the fertilized chicken egg at HH 40-42. CAM tissue was washed twice with PBS (pH 7.4), then fixed with 4% PFA, 4% sucrose in PBS for 20-40 minutes, permeabilized with 0.2% TritonX-100/PBS solution for 30 minutes and blocked with blocking solution (1% goat serum, 2% BSA, 0.1% sodium acid, 0.2% TritonX-100) for 1 hour. Primary and secondary antibodies were diluted in blocking solution and incubated overnight at 4°C. In between and after the incubation with antibodies, CAM tissue was washed three times with PBS for 10-30 minutes. CAM tissue was covered with ProLong Gold antifade reagent (Invitrogen), and images were acquired using a Leica SP5 inverse laser confocal microscope (Microsystems GmbH) equipped with 20/40/63x objectives. The fluorescence channels were separately acquired by a sequential scanning mode.

**FIGURE 1** Expression and localization of Cxs in the CAM. A, Qualitative RT-PCR revealed mRNA expression of GJA4 (Cx37), GJA5 (encoding for Cx40 in mammals and Cx42 in chicken), and GJA1 (Cx43) in CAM tissue. beta-Actin (ActB) served as loading control. B, Immunoblotting confirmed protein expression of Cx40/42 and Cx43. Cx37 was not found in CAM lysates. To test for Cx40/42 expression, two different antibodies were used (see section 2 and Table 2). The antibody targeting the N-terminal region of human Cx40 (a) consistently detected Cx40/42, whereas the one designed against an internal sequence of human Cx40 (b) did not. C, Representative immunofluorescence pictures of CAM vascular networks. Staining with the pericyte/smooth muscle cell marker desmin allowed tracking of arterial and venous vessels (upper panel). Staining for Cx43 revealed localization in ART (filled arrows) at the membrane of neighboring cells (detail 1, 2, and merged image). In VEN (open arrows), the signal was not specific (detail 1 and merged image). Cx40/42 was not detected by CFM. CAMs of n = 3 chick embryos were used for all experiments.
2.7 | Cell culture

The permanent somatic endothelial cell line EA.hy926 (ATCC CRL-2922) was maintained in DMEM (Biochrom AG) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin and cultured at 37°C in a humidified atmosphere of 5% CO2. Cell culture medium was changed every second day, and once a week, the cells were passaged using 1% Trypsin/EDTA.

2.8 | Determination of cytotoxicity

EA.hy926 cells were seeded at a density of 4000 cells/well into 96-well microtiter plates and incubated with the unspecific GJs blockers CBX (50, 175, 400 µmol/L) and PA (100 µmol/L) for 1-6 hours. One hundred microliter of each sample was incubated with 100 µL LDH assay reagent for 10 minutes at room temperature in the dark. Release of LDH was determined by a colorimetric kit as described previously.27 Extinction (E) was measured at 490 nm. Cytotoxicity of a specific compound was calculated as

\[
\text{Cytotoxicity}(\%) = \frac{E_{\text{compound}} - E_{\text{low control}}}{E_{\text{high control}} - E_{\text{low control}}} \times 100
\]

2.9 | Pharmacological modulation of gap junction function

Effects of the following GJ blockers (all purchased from Sigma Aldrich) on arterial and venous vessel diameters were determined:

- Carbenoxolone—the glycyrrhizin acid metabolite CBX is a non-specific inhibitor of intercellular communication commonly applied in concentrations of about 100 µmol/L.28-30 Here, we used increasing concentrations from 25 to 400 µmol/L.
- Palmitoleic acid—the fatty acid PA, which disrupts GJ communication by intercalation in the lipid bilayer, blocks Cx40 and Cx43 in working concentrations up to 50 µmol/L.31-33 Since PA is poorly water-soluble and easily oxidized, working solutions (100 µmol/L) were prepared under nitrogen atmosphere out of PA stock solutions (100 mmol/L) in DMSO. The concentration of DMSO in working solutions was 0.1%.
- GAP27—the GJ blocking peptide GAP27 mimics amino acids 204-214 (SRPTEKTIFII) on the extracellular loop 2 of Cx43 blocking GJ communication by disturbing connexon assembly.26 The SRPTEKTIFII

![Figure 2](image-url)
sequence is completely conserved in chick Cx43, except that isoleucine (position 214 in human Cx43) is replaced by valine, which is also a non-polar amino acid like isoleucine. GAP27 concentration used for experiments was 1000 µmol/L. Combination of GJ blocking peptides may increase their efficacy.\textsuperscript{34} Here, however, only one peptide was used.

All GJ blockers were dissolved in PBS and applied every 2 hours (t = 0, 2, 4 hours) in drops of 25 µL to warrant a permanent coverage of the investigated CAM areas.

Additionally, we studied the effects of the GJ function stimulating agent DIP. DIP increases the concentration of cytosolic cAMP which is believed to increase GJ coupling via different pathways such as elevated Cx trafficking, changes in phosphorylation status and increased Cx expression.\textsuperscript{35} Chick chorioallantoic membrane vasculature was exposed for 24 hours to PBS (control), 175 µmol/L CBX, 50 µmol/L DIP, or a combination of CBX and DIP for 24 hours. For these experiments, 20 µL of 175 µmol/L CBX was applied topically at the beginning of the experiment. DIP was dissolved in DMSO (10 mmol/L) and diluted in PBS at 1:200.

\subsection{2.10 Vascular tone of CAM vessels}
In order to determine VT of arteriolar and venous CAM vessels, 75 µL of a vasodilator cocktail consisting of 10 µmol/L Ach (Sigma Aldrich), 100 µmol/L ADO (Serva Feinbiochemica), 200 µmol/L PAP (Sigma Aldrich), and 10 µmol/L SNP (Sigma) dissolved in PBS was applied on either native (for determination of resting tone) or CBX-treated CAM vessels for 15 minutes to achieve maximal vessel dilatation. Vascular tone was expressed as

\[ VT = \frac{D_{\text{max}} - D}{D_{\text{max}}} \]

where \( D_{\text{max}} \) is the maximal diameter after application of the dilatory cocktail and \( D \) is the vessel diameter before dilatation (resting diameter or diameter after CBX treatment, respectively).

\subsection{2.11 Statistical analysis}
For RT-PCR, Western Blotting and immunofluorescence, at least \( n = 3 \) CAMs per experiment were used. For each substance and/or concentration applied on CAM vessels, a minimum of \( N = 20 \) ART and 20 VEN were analyzed. If not stated otherwise, each CAM experiment was independently reproduced three times (\( n = 3 \) CAMs). Data are given as mean ± SEM. Diameter changes were analyzed using Student’s t test; a P-value < .05 was considered significant.

\section{RESULTS}

\subsection{3.1 Connexin 43 is abundantly expressed in arterial CAM vessels}
In order to screen for Cxs potentially expressed in CAM tissue, we performed RT-PCR which revealed mRNA expression of Cx37 (GJA1), Cx40/42 (GJA5), and Cx43 (GJA4; Figure 1A).

\subsection{3.2 Connexin blockers cause arterial and venous vessel diameter decrease}
To test whether the observed protein expression had functional relevance in intact CAM vasculature, we treated CAM networks with the non-specific Cx blockers CBX and PA as well as the Cx43 specific blocker GAP27 (Figure 2A). All inhibitors decreased vessel diameters in both ART and VEN (Figure 2B).
CBX-induced vessel diameter reduction was abolished suggesting that the observed effects are mediated by changes in Cx function rather than being non-specific.

3.4 CBX-induced vessel diameter decrease is mediated by tone-dependent and tone-independent mechanisms

In order to evaluate the role of VT before and during Cx blockade, we applied a vasodilator cocktail consisting of Ach, ADO, PAP, and SNP on untreated and CBX-treated CAM networks (Figure 6).

Vascular tone of arterial (10 ± 1%) and venous vessels (6 ± 4%) was low under resting conditions. Initially, increase of VT contributed significantly to the CBX-induced vessel diameter reduction: more than half of the diameter decrease of ART and VEN that occurred within the first 3 hours after CBX treatment could be reversed by maximal vasodilatation. After 6 hours of CBX treatment, however, vasodilatory responses were strongly reduced, indicating that diameter reduction had become structurally fixed.

4 DISCUSSION

It has been extensively shown that vascular GJs provide the basis for the propagation of local vasodilatory and vasoconstrictive stimuli by allowing the passage of ions and small molecules between adjacent cells of the vessel wall.13,36,37 However, most of our knowledge on vascular GJ function is deduced from in vitro studies or investigations of single blood vessels, ignoring the interdependency of coupled blood vessels in microvascular networks. Also, a little is known about the importance of GJs for mediating prolonged vascular changes, as most of the studies have investigated their role as mediators of acute vasomotor responses.

In this study, we examined the role of Cxs for the acute and prolonged regulation of vessel diameter in functional networks of the CAM vasculature, the avian homologue of human fetoplacental circulation. This in vivo model allows live observation for extended durations of up to 24 hours and thus tracking of sustained and structural changes.

We found that Cx43 is ubiquitously expressed in arterial but not venous CAM vessel walls (Figure 1C) and that Cx blockade with the non-specific inhibitors CBX, PA as well as the specific inhibitor GAP27 led to diameter decrease in both arterial and venous vessels (Figure 2). This effect is likely to be specific to Cx blockade since none of the non-specific blockers showed relevant cytotoxicity on EA.hy926 endothelial cells (Figure 4), and vessel diameter reduction under CBX treatment was abolished in the presence of the GJ stimulating agent DIP (Figure 5). The contribution of increased VT was relevant for the early phase up to about 3 hours but decreased strongly after 6 hours, suggesting an increasing structural fixation of diameter decrease (Figure 6).

The local expression pattern of vascular Cxs differs between different blood vessel types, different cells of the vascular wall, and...
To our knowledge, Cx expression has not been investigated in CAM vasculature so far and it is surprising that Cx43, and not Cx40/42, seems primarily responsible for mediating conducted responses in CAM vessels. From the known functions of Cxs and the present observations in the CAM, the following hypotheses may be derived:

- Gap junctions are abundantly expressed on the arterial side of CAM vasculature to ensure propagation of vasodilatory stimuli, putatively generated at the capillary level.
- These stimuli are needed to keep VT of upstream arterial vessels at a low level and thus prevent acute arterial flow reduction and long-term regression or even collapse of these vessels.
- Since arterial and venous vessels are hemodynamically coupled, arterial diameter decrease also causes reduced pressure, volume flow, and shear stress in venous vessels resulting in attenuated release of vasodilatory substances, that is, NO, and increased VT and structural diameter decrease.

If vessel diameter reduction exceeds a certain threshold, blood flow is impaired. For CBX concentrations of 175 µmol/L or higher, blood flow stasis was observed in both arterial and venous vessels (Figure 3). Since flow resistance is inversely dependent on the fourth power of vessel diameter (Poiseuille's law), vessel diameter reduction of approximately 50% as seen under treatment with 175 µmol/L CBX causes a 16-fold increase of blood flow resistance which leads to malperfusion and eventually blood flow stasis in the supplied area of the CAM.

Previous studies have investigated the relationship between blood flow velocity, mechanical stress, and Cx43 expression. It has been reported, that Cx43 mRNA increased in endothelial cells subjected to sustained levels of mechanical strain and fluid shear stress. Another study by DePaola et al. showed that endothelial Cx43 protein expression increased and decreased with increasing and decreasing blood flow levels. With regard to our data, it would be intriguing to analyze blood flow velocity, wall shear stress, and Cx43 protein expression in CAM vessels before and after short-term and long-term GJ blockade. Further studies are needed to clarify these aspects.

The above-mentioned observations might have different clinical implications. Maldistribution and functional shunting have been identified as pathophysiologic driving forces during shock or ischemia-reperfusion. In this context, it was also shown that bacterial lipopolysaccharides, an initiating factor in sepsis, attenuate cell-to-cell communication, most likely by altering the phosphorylation status of different Cxs. With the limitation that the experimental setup provided here cannot mimic the complex conditions leading to sepsis, our data give in vivo evidence that break-down of gap junctional function results in microcirculatory changes similar to those seen in sepsis (malperfusion, hypoxia) and maybe even disseminated intravascular coagulation (stasis/thrombosis).
Tumor microvascular networks are characterized by high structural and functional heterogeneity in which excessively and poorly perfused tissue areas coexist in close neighborhood. Considering in vitro data—VEGF, a proangiogenic factor elevated in most solid tumor, was shown to block endothelial cell–cell communication and results from mathematical modeling our group hypothesized that impaired GJ-based intercellular communication leading to functional shunting plays a significant role in this process. Although the formation of direct shunt pathways in response to GJ blockade could not be observed in this study, malperfusion might still be related to functional shunting. CAM blood flow could pass by network areas deprived of GJ communication using the remaining pathways mirroring systemic shunting. With regard to tumor tissue, loss of GJ function might cause malperfused and hypoxic tissue areas prone to genetic instability, metastasis and probably even further dysfunction of GJs (eg, hypoxia results in the loss of Cx43 function in the myocardium).

Blood vessels of vascular beds responsible for oxygen uptake (eg, pulmonary or fetoplacental beds like the CAM) constrict in response to hypoxia. In this context, we have reported an essential role for Cxs in the mediation of hypoxic pulmonary vasoconstriction. Our present observations suggest that the role of GJs in fetoplacental beds such as the CAM expands beyond mediating vasoconstrictive responses.

Firstly, GJs are responsible for transferring vasodilatory signals from capillaries to arterioles in order to maintain low-resting tone of these vessels (ART 10 ± 1%, VEN 6 ± 4%, Figure 6). This reflects the function of the CAM as a facilitator of maximal gas exchange for a continuously growing embryo. Varying functional demands caused by different states of rest and work that occur in striated muscular tissue, for example, do not exist in the CAM. This removes the need for high-resting tones that allow rapid up- and downregulation of blood flow. By properly adapting vessel tone to the functional demand of the organism, GJs might also play a role in optimizing the energetic cost of vascular networks. De Wit et al pointed out that information transfer via GJs is critical for the matching of oxygen delivery and tissue needs in a well-tuned fashion.

Secondly, GJs play a role in chronic remodeling of CAM vessels. After 6 hours of incubation with CBX, vessel reaction upon vasodilatation declines, suggesting structural remodeling of the vascular bed. This finding is in agreement with studies performed by Bakker and Van Bavel who demonstrated that long-term increase of VT is a major driving force in vessel remodeling by structural adaptation. Similarly, acute hypoxia results in vasoconstriction in the lung, while chronic hypoxia causes vascular remodeling and vessel rarefaction. However, several important aspects of this remodeling process are still unclear. First, the underlying molecular mechanisms that trigger vascular remodeling in the CAM (eg, changes in calcium signaling) remain to be elucidated. Secondly, the CX distribution data suggest that a direct, Cx-mediated change in tone and structure should be restricted to the arteriolar side of CAM vasculature but observed vessel diameter changes are equally strong on the venous side. Based on previous studies on vascular reaction to reduced perfusion including simulation studies of structural vascular adaptation, it may be assumed that changes on the venular side reflect...
the reduction of blood flow through the CAM network. This concept demands further investigations including histological and modeling approaches.

**PERSPECTIVE**

In summary, GJs are important for network homeostasis by acutely regulating vessel tone and by chronically regulating vascular structure and vessel remodeling. The molecular mechanisms by which gap-junctional communication orchestrates these processes remain to be elucidated. Our data provide novel insight into the complex role of GJs in the regulation and adaptation of vascular networks in vivo and expand our knowledge on systemic vascular adaptation.

**DISCLOSURE**

The authors declare no conflict of interest.

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