MINI REVIEW

Endothelial connexins in vascular function

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Abstract

Gap junctions are essential for intercellular crosstalk in blood and lymphatic vasculature. These clusters of intercellular channels ensure direct communication among endothelial cells and between endothelial and smooth muscle cells, and the synchronization of their behavior along the vascular tree. Gap junction channels are formed by connexins; six connexins form a connexon or hemichannel and the docking of two connexons result in a full gap junction channel allowing for the exchange of ions and small metabolites between neighboring cells. Recent evidence indicates that the intracellular domains of connexins may also function as an interaction platform (interactome) for other proteins, thereby regulating their function. Interestingly, fragments of Cx proteins generated by alternative internal translation were recently described, although their functions in the vascular wall remain to be uncovered. Variations in connexin expression are observed along different types of blood and lymphatic vessels; the most commonly found endothelial connexins are Cx37, Cx40, Cx43 and Cx47. Physiological studies on connexin-knockout mice demonstrated the essential roles of these channel-forming proteins in the coordination of vasomotor activity, endothelial permeability and inflammation, angiogenesis and in the maintenance of fluid balance in the body.

Key Words
▶ gap junction
▶ connexin
▶ endothelium
▶ intercellular communication

Introduction

Intercellular communication is important for vascular function and homeostasis. Connexins (Cxs) play an essential role in this crosstalk. Cxs are ubiquitous transmembrane proteins, which have the ability to form hemichannels at the plasma membrane and intercellular channels called gap junctions (GJs) connecting the cytoplasms of two neighboring cells (Fig. 1). Cxs are expressed in virtually all tissues and cell types (1, 2), including endothelial cells (ECs) and smooth muscle cells (SMCs) of blood and lymphatic vessels (3).

Twenty-one different Cx genes have been described in the human genome and 20 genes in the murine genome, encoding for Cx proteins with a molecular weight between 20 and 62 kDa. All Cxs share the same protein topology with four transmembrane domains (TM1-4), two extracellular loops (EL1-2), a cytoplasmic loop (CL) and N- and C-terminal domains (NT and CT). Cxs are synthesized in the endoplasmic reticulum (ER) and oligomerize in hexameric connexons in the ER/Golgi or trans-Golgi network (1). Then, connexons travel by vesicular carriers along microtubules to the plasma membrane, where these hemichannels have a finite, very low, open probability under physiological conditions. The open probability of hemichannels is increased in response to specific (often pathological) stimuli, providing a pathway for transmembrane signaling (Fig. 1). When plasma membranes of two cells are in close proximity, connexons from one cell can dock with their equivalents in the adjacent cell to form GJ channels that enable a direct exchange of ions and metabolites between cells (Fig. 1).
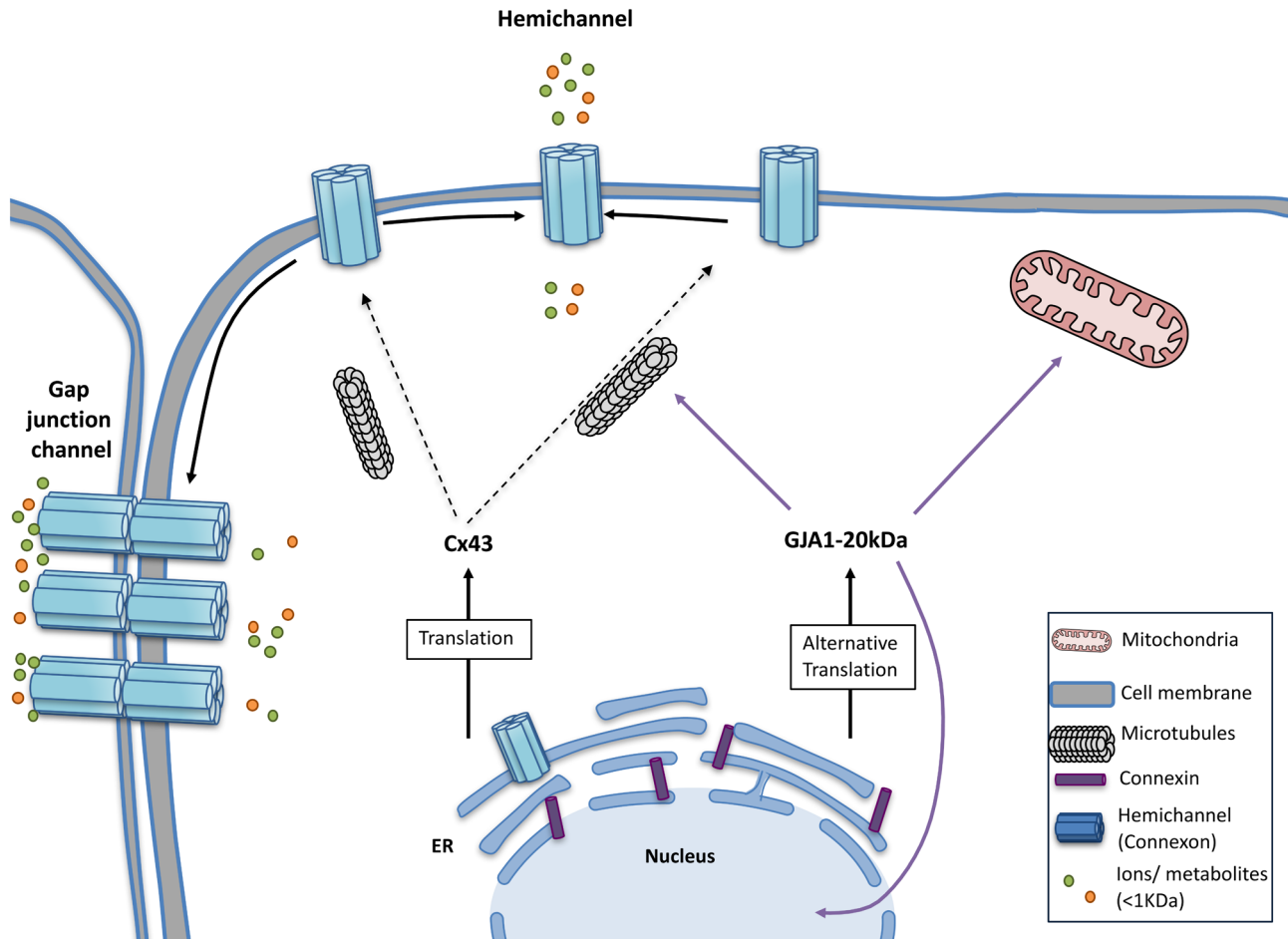
Figure 1

Synthesis and trafficking of Cxs. After translation, Cxs are inserted in the endoplasmic reticulum (ER) and then assemble into hexameric connexons in the ER or Golgi apparatus. Connexons travel across microtubules to the cell surface where these hemichannels remain closed or may be opened in response to pathological stimuli. Connexons from adjacent cells pair to form GJ channels allowing the direct exchange of ion or metabolites between both cells. Alternative internal translation of the *GJA1* gene transcript (encoding for Cx43) produces a 20 kDa fragment representing the C-terminal 169 amino acids of the protein. GJA1-20 kDa has been shown to stabilize actin filaments to guide full-length Cx43 delivery to the plasma membrane, to facilitate microtubule-based mitochondrial transport, and it has been shown to translocate to the nucleus of cells.

In addition to their roles in GJs and hemichannels, emerging data points to channel-independent roles for Cx in tissue homeostasis and disease. Such non-canonical functions are best described for Cx43 and include, for instance, the regulation of gene transcription through transcription factor hijacking, or acting as a scaffolding protein to regulate the dynamics of other proteins at the cell membrane (2, 4). In this context, the recent discovery of Cx43 fragments generated by internal (alternative) translation is of particular interest (4). Finally, mutations or polymorphisms in Cx genes have been associated with various cardiovascular diseases such as atrial fibrillation, hypertension, atherosclerosis, thrombosis and lymphedema (5). In this brief review, we will focus on the physiological role of Cxs in cell-cell communication in the vasculature, with particular attention for EC-EC and EC-SMC crosstalk.

Cx expression in various regions of the vascular tree

In the vascular wall of blood vessels, four Cxs have been found in ECs and SMCs, specifically Cx37, Cx40, Cx43 and Cx45. The size and abundance of GJs as well as the expression patterns of individual Cxs has been shown to vary in different regions of the vascular tree and with the progression of disease states such as atherosclerosis and hypertension.

Large elastic arteries such as the aorta distribute the blood from the heart into the arterial system and dampen the pulsatile pressure that results from the intermittent ejection of blood from the left ventricle. Large muscular arteries subsequently distribute the blood flow to specific territories or organs. Although large distributing arteries

are capable of constricting and dilating, they have no significant role in the regulation of blood flow to organs or blood pressure under physiological conditions. Cx expression has been mostly studied in such arteries in the context of atherosclerosis, a slowly progressing disease with chronic inflammation and lipid accumulation in the wall of large- and medium-sized arteries at sites of blood flow disturbance. In general, Cx37 and Cx40 are abundantly expressed in ECs of large arteries, whereas Cx43 and Cx45 are found in their SMCs (2, 3). Detailed *en face* immunofluorescence studies revealed that Cx37 and Cx40 are highly expressed in ECs of straight parts of arteries that are exposed to high laminar shear stress and that these Cxs are downregulated in ECs at arterial branch points or bifurcations subjected to a disturbed blood flow (6, 7, 8). In contrast, the expression of Cx43 is induced in ECs exposed to disturbed flow (3, 7), as illustrated in Fig. 2 for the carotid bifurcation. Although intercellular transfer of Lucifer Yellow has been demonstrated between ECs in large arteries (9), much of the Cx43 expressed in ECs exposed to disturbed flow is not located at sites of cell–cell contact (Fig. 2), suggesting that it may fulfill a non-canonical function at these sites. There is increasing evidence supporting GJ-independent functions of Cx43 that only require the expression of Cx43-CT fragments (2, 10). For instance, Cx43-CT has been shown to regulate cytoskeletal conformation, proliferation, migration and differentiation in a variety of cells. *In vivo*, Cx43-CT mediates neuroprotection during stroke (11). Although 20 kDa immunoreactive bands have long been detected in Western blots of cell lysates of various origin, only recently was compelling evidence provided that internal translation of the *GJA1* gene transcript (encoding for Cx43) produces a 20 kDa fragment, thus allowing for increased protein diversity from a single mRNA transcript (12, 13). This GJA1-20k fragment (representing the C-terminal 169 amino acids of the protein) has been shown to stabilize actin filaments to guide full-length Cx43 delivery to cardiac intercalated discs (14), to facilitate microtubule-based mitochondrial transport during cellular stress (15)

and to restrict gap junction formation during epithelial/ mesenchymal transition (16). Whether the GJA1-20k isoform exists in ECs and whether it may fulfill comparable roles in ECs exposed to disturbed shear stress is presently unknown. Although it is certainly tempting to follow-up on such new hypotheses, it should still be kept in mind that dye transfer varied between straight parts and branch points of arteries, indicating not only functionality but also specificity of GJ intercellular communication (GJIC) in these arterial regions (8).

Small muscular arteries and arterioles regulate arterial blood pressure and blood flow within organs. They can actively alter their vessel diameter in response to autonomic (sympathetic) nerve activity, circulating hormones (*e.g.* catecholamines, angiotensin II) or metabolites produced by the tissues surrounding the vessel or by the vascular endothelium itself (*e.g.* adenosine, K^+, nitric oxide (NO)). Myoendothelial gap junctions (MEJs) play an important role in the intercellular passage of EC-generated vasodilating signals. Although some differences in arteriolar Cx expression have been described depending on the vascular bed and the species studied, in general Cx40, Cx37 and Cx43 are found between ECs, Cx43 and Cx45 (and sometimes Cx40 or Cx37) between SMCs, and Cx43 and Cx40 or Cx37 likely form MEJs (17, 18).

In capillaries, the primary exchange vessels within the body, Cx37 and Cx43 are typically present between ECs. Finally, Cx43 and Cx37 have been identified in ECs of (non-valved) veins (19). Interestingly, ECs on venous valves show a particular Cx expression pattern with Cx43 at upstream sides, Cx37 at downstream sides and Cx47 being restricted to a specific subset of cells composing the valves (20, 21). A similar valve side-specific expression pattern has been observed for Cxs in ECs covering lymphatic valves (22, 23). Lymphatic SMCs express Cx45 (24).

The endothelium is the unique interface between vascular lumen and the rest of the vessel wall. In addition to its role as a physical barrier, it is a major actor in vascular homeostasis. As already briefly mentioned above,
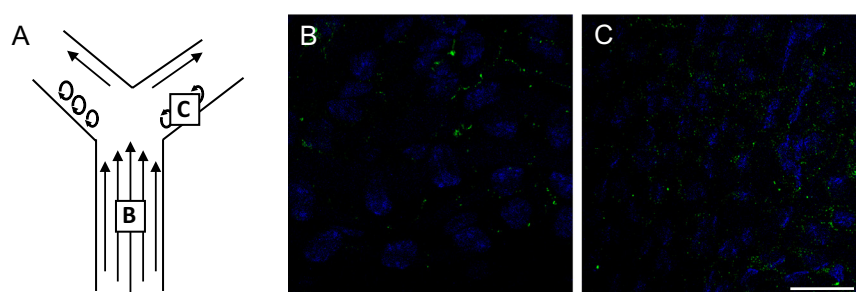

Figure 2
Endothelial Cx43 expression is increased in arterial regions exposed to disturbed flow. (A) Schematic representation of arterial regions exposed to unidirectional and disturbed flow. (B) *En face* immunostainings of Cx43 (in green) in mouse arterial endothelium of the common carotid artery (b) and at the carotid bifurcation (C). Nuclei are stained with DAPI (in blue). Scare bar represents 30 μm.

physical (shear stress due to blood flow) or molecular stimuli (neurotransmitters, circulating hormones, metabolites) from the blood or in the surrounding tissues will induce secretion of products from ECs that modulate vascular tone, leukocyte adhesion or angiogenesis, for example. Cxs are involved in these processes by allowing GJIC and thus coordinated signal spread through the vessel wall as well as by regulating other signaling pathways through the Cx interactome. Additionally, Cx fragments derived from alternative (internal) translation may also participate in this process.

Coordination of vasomotor reactivity by GJs

The endothelium plays an important role in the control of vascular tone by Ca^{2+}-dependent production of vasodilatory signals such as nitric oxide (NO) and prostaglandins. GJs have appeared to be a key pathway to control blood flow distribution by resistance arteries by permitting radial (EC-SMC) and longitudinal (along the vessel length) conduction of vasomotor signals (25, 26, 27).

Although the internal elastic lamina mostly separates ECs and SMCs, these cells reach each other at discrete points of contact, called MEJs, which constitute highly specialized subcellular signaling domains. GJs located at MEJs critically regulate vasomotor responses through the radial transmission of current, Ca^{2+} and small signaling molecules like inositol triphosphate (IP_3). In fact, a prominent component of endothelium-derived hyperpolarizing factor (EDHF) signaling is now thought to result from direct electrotonic transmission of a hyperpolarizing current initiated by endothelial Ca^{2+}-activated K^+ channels via MEJs. In agreement, EDHF vasodilatory signaling is attenuated by Cx-mimetic blocking peptides directed against Cx43, Cx40 or Cx37 (28). Furthermore, EC-specific deletion of Cx40 also reduces signaling via the EDHF pathway (29). Although NO may freely diffuse across cell membranes, GJ-mediated NO signaling at MEJs appeared to play a role in acetylcholine (ACh)-induced NO-dependent vasodilation in resistance arteries (30). The Cx involved in the radial transfer of vasodilatory signal remains however to be identified.

Longitudinal conduction of vasomotor signals contributes to the coordinated response and integrated function within an arteriolar network or between arterioles and feeding arteries. Conducted vasomotor responses are thought to result from the electrotonic spread of changes in membrane potential through GJs from the site of origin along the vessel length to upstream or downstream vessel segments. The contribution of each endothelial Cx to the conduction of vasomotor responses is not yet completely resolved. However, *Cx40$^{-/-}$* mice show an irregular arteriolar vasomotion and a reduced spread of ACh-induced or bradykinin-induced vasodilatory responses into feeding arteries (31, 32, 33). Reduced propagation of vasodilatory responses was also observed in mice with EC-specific deletion of Cx40, certifying the involvement of endothelial Cxs in the longitudinal conduction of these responses. Finally, Cx40 deficiency in mouse endothelium is accompanied by a reduction in Cx37 expression (34, 35); however, the conducted vasodilatory response is intact in *Cx37$^{-/-}$* mice (31, 33), thus further attesting to the crucial role of endothelial Cx40 in this physiological process.

Involvement of Cxs in endothelial permeability and inflammation

Disruption of the endothelial barrier function resulting in increased vascular permeability is a key event in pathological conditions associated with edema and inflammation. Although the endothelial barrier is principally regulated by tight junctions (TJs) sealing the intercellular space and adherens junctions (AJs) providing mechanical anchorage (36), recent evidence suggests that GJs and/or Cxs may play a role in the regulation of the endothelial barrier function as well (37).

Cx40 is abundantly expressed in the lung vasculature and has been demonstrated to play critical roles in endothelial homeostasis and the regulation of endothelial barrier function, albeit with different effects on inflammation and edema (34, 38). On the one hand, lung edema and injury were attenuated in *Cx40$^{-/-}$* mice after induction of acute lung injury (ALI) by acid or lipopolysaccharides (LPS) instillation. In fact, Cx40 appeared to play a role in the regulation of Rho-associated protein kinase 1 (ROCK1) and myosin light chain 20 (MLC_{20}), a pathway that contributes critically to lung endothelial permeability (38). On the other hand, endothelial-specific ablation of Cx40-mediated GJIC enhanced neutrophil recruitment in a murine model of ALI by perturbing the propagation of adenosine-evoked anti-inflammatory signals between ECs (34). This mechanism seems to regulate neutrophil recruitment in cardiac ischemia/reperfusion injury (39) and monocyte recruitment in the context of atherosclerosis as well (34). Interestingly, another endothelial GJ protein, namely

Cx43, seems to regulate the spread on pro-inflammatory signals in the lung capillary bed (40). Indeed, endothelial Cx43 mediates acid-induced increases in pulmonary microvascular permeability (40) and it modulates neutrophil recruitment to the lung after LPS instillation (41). Furthermore, it has been suggested that osteopontin (OPN) participates in the regulation of vascular permeability by Cx43 after sepsis (42). The authors described that Cx43 upregulates OPN via the transcription factor 4 (Tcf-4)/β-catenin transcription pathway. OPN subsequently increases vascular permeability by downregulating the expression of the TJ proteins zonula occludens-1 (ZO-1) and claudin-5 (42). In addition, inhibition of endothelial Cx43 has been shown to blunt vascular hyperpermeability during recovery from lung injury via effects on the expression of the AJ protein VE cadherin (43). Whether Cx43 also acts as a critical regulator of TJ or AJ proteins in arteries or in other microvascular beds remains to be investigated. Of particular interest in this context is a recent study demonstrating that Cx43 gap junctions contribute to brain endothelial barrier hyperpermeability in familial cerebral cavernous malformations type III (fCCM3) by modulating the structure of TJs (44).

Cytokine-induced opening of Cx43 hemichannels has been implicated in multiple inflammatory diseases (45). Mechanistically, hemichannels appeared to regulate the amplification and perpetuation of inflammation by mediating an ATP autocrine feedback loop in the inflammasome/inflammation cycle (46). In consequence, targeting Cx43 hemichannels may offer an attractive therapeutic strategy for acute and chronic inflammatory diseases, and a variety of Cx43-based channel blockers have been developed in recent years. Unfortunately, most of these blockers display either poor Cx isoform specificity or they inhibit GJ channels and hemichannels at the same time (2). More promising specific Cx43 hemichannel blockers include the peptide Gap19 (47) and Peptide5 (48). However, the increased expression and cytokine-induced opening of Pannexin1 channels, a distinct family of ATP release channels (49), during inflammation closely resembles the ones of Cx43 hemichannels (45). Future research should aim to better discriminate between the functions of these protein families in inflammatory disease.

Connexins in angiogenesis

Angiogenesis is a primordial process for remediating local hypoxia during wound healing or tissue regeneration.

During angiogenesis, new capillaries sprout from pre-existing blood vessels to expand the vascular plexus. The primary stimuli are a lack of oxygen and nutrients in the surrounding tissues, which induce the production of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), by non-vascular cells. This will activate the normally quiescent ECs to start proliferating and migrating (50). The implication of GJs in the proliferation of tumor cells in cancer has been intensely studied in the past 50 years (51); however, much less is known about the role of Cxs in postnatal angiogenesis. Knockdown of endothelial Cx43, Cx40 or Cx37 in 3D Matrigel *in vitro* angiogenesis assays induces a pronounced effect on capillary network formation with a reduced number of branches and branches to capillary–length ratio (52). Adult $Cx37^{-/-}$ mice show improved recovery of hindlimb ischemia, likely due to a growth-suppressive action of Cx37 in ECs, and thereby limiting angiogenic and arteriogenic responses to ischemic injury in the adult animals (53, 54). Interestingly, Cx37 appeared critical in the formation of vascular networks as a component of a mechano-chemical pathway in which arterial shear stress activates a NOTCH-Cx37-CDKN1B signaling axis that promotes EC cycle arrest and enables arterial specification (55). Targeting Cx40 *in vivo* reduces angiogenesis in the developing mouse retina (56) and inhibits tumor growth by reducing angiogenesis (57). In an unbiased transcriptomics approach, cell cycle progression was identified as an important downstream target of Cx40 under arterial level of wall shear stress (58), illustrating that the regulation of proliferation by Cx40 may well extend beyond capillary ECs. Moreover, zebrafish embryos knockout for the Cx40 orthologues *Cx41.8* and *Cx45.6* displayed faster intersegmental vessel growth and branching into the dorsal longitudinal anastomotic vessels, an effect that was associated with increased EC proliferation (58). This suggests that the close relation between Cx40 and EC proliferation in sprouting angiogenesis is probably well conserved during evolution.

Connexins in lymphatic edema

Lymphedema is a condition with localized extracellular fluid retention and tissue swelling due to a compromised lymphatic system. Lymphedema may be acquired after cancer treatment or parasitic infections for example (secondary lymphedema), but it may also be caused by gene mutations (primary lymphedema). Mutations in Cx47 (*GJC2*), Cx43 (*GJA1*) and more recently Cx37 (*GJA4*) genes cause human lymphedema (5, 59, 60).

Cx37, Cx43 and Cx47 are all expressed in lymphatic ECs (22, 23). *Cx37⁻/⁻* mice show perturbed lymphatic valve formation resulting in virtual absence of valves and significant lymphedema in adults (22, 23). Mice with a lymphatic EC-specific ablation of Cx43 show a delayed initiation, reduced frequency and incomplete maturation of lymphatic valves and altered lymphatic capillary patterning, resulting in leaky valves, insufficient lymphatic transport and a high incidence of lethal chylothorax, a milky effusion around the heart and lungs, in the first 6 months of life (61). Although mutations in the *GJC2* gene predispose for human lymphedema, Cx47 expression is only sporadically detected in lymphatic ECs of the embryonic and adult mouse (22, 62). Moreover, Cx47 deficiency did not affect lymphatic contractility or morphology nor interstitial fluid drainage in adult mice (62). It has recently been reported that human venous valve disease may be caused by mutations in the *GJC2* gene. Patients with such mutation show reduced number and length of venous valves (20). Interestingly, *Cx47⁻/⁻* mice display disrupted organization and reduced proliferation of venous valve-forming ECs, which may underlie the venous valve defects seen in patients with *GJC2* mutations (20). Comparable changes in venous valve-forming ECs were observed in *Cx37⁻/⁻* and *Cx43⁻/⁻* mouse embryos, suggesting that it might be relevant to search for mutations in *GJA4* and *GJA1* genes in venous valve disease patients.

Conclusions

Research on Cx-deficient mice in the past two decades has demonstrated an essential role for these proteins in vascular pathophysiology. As described in the previous sections, coordination of vasomotor activity, endothelial permeability and inflammation, angiogenesis and the maintenance of fluid balance in the body critically depend on the presence of Cxs, either in GJs or as connexons at the cell membrane. Future challenges now include the development of specific Cx-targeting therapies for treatment of cardiovascular disease. Major obstacles that are currently encountered are the specificity of small molecules, peptides or antibodies for individual Cxs or for GJ- vs hemi-channels.

It is becoming increasingly clear that Cxs regulate vascular pathophysiology not only through their channel-forming abilities, but can also control vascular function through their interaction with other proteins. In the endothelium, Cx37 binds to the endothelial nitric

oxide synthase (eNOS) and the interaction between these proteins seems to affect both Cx37 channel gating as well as eNOS enzymatic activity (63). Moreover, Cx40 has been shown to interact with IκBα. Their binding dampens NFκB nuclear translocation and, in consequence, endothelial activation (6). Many more players of the endothelial Cx interactome remain to be identified in the years to come. Finally, naturally occurring C-terminal isoforms generated via alternative internal translation of Cx43 have recently been discovered and regulate critical aspects of cardiac physiology (14). The function of this Cx43-20k isoform has not yet been explored in the context of vascular physiology and disease and leaves the research field with much new and exciting work ahead.

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