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REVIEW ARTICLE

A Review of the Current State of Pannexin Channels as They Relate to the Blood Vessel Wall

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Pannexin proteins comprise a family of channels whose sole function, to date, is the release of nucleotides (e.g., adenosine 5'-triphosphate [ATP] and uridine 5′-triphosphate [UTP]). With purinergic signaling being such a prevalent form of cellular communication, it is hard to image why a channel dedicated to the release of nucleotides has not been previously identified. However, with their topography and discovery being lumped with the gap junction field (i.e., connexin), they were thought for a long time to be more similar to connexin-based proteins. It is now known that there is a distinct difference between pannexins and connexins. Unlike connexin hemichannels (undocked gap junctions), pannexins can open under physiological Ca^{2+} levels. With their distribution being nearly ubiquitous across the vasculature and importance of purinergic signaling in the vasculature, it is easy to see why pannexin channels may be, especially, important. In this mini-review, we highlight what we know about the cell biology of pannexins, followed closely by what is known about pannexins in the vasculature in regards to its importance in vascular physiology.

Key words: Pannexin, connexion, blood vessels

CELL BIOLOGY OF PANNEXINS

All pannexins possess a similar structure with four transmembrane domains, two extracellular loops, an intracellular loop, and both the amino and carboxyl termini located intracellularly.[1] Generally, six pannexins come together and form a channel, except for Panx2 that seems to form heptamers or octamers.[2] There is limited evidence in vertebrates for pannexin channels to dock with channels of neighboring cells to form functional pannexin-formed gap junction channels.[3]

Pannexins are expressed throughout the cardiovascular system. The expression levels within the vascular tree are dependent on vessel type, localization within the vessel, and the species being examined. The expression differences between macrovessels versus microvessels and arteries versus veins are continuing to be examined; however, the specificity of antibodies and detection of low expression levels remains limiting steps to overcome. The majority of the data of pannexin isoforms and their unique localizations along the vascular tree comes from the studies using rodent models or human primary cells. Thus, species-dependent differences also add another level of complexity to our understanding of the localization and function of these isoforms within the vascular wall.

Pannexin 1 is ubiquitously expressed in murine endothelial cells (ECs) within arteries, arterioles, and venules, as well as in isolated human cells including an immortalized brain EC line, human saphenous vein ECs, and human umbilical vein ECs (HUVECs).[4-6] Panx1 expression in murine smooth muscle cells (SMCs) occurs only in arterioles and venules, whereas Panx3 is only found in coronary arterioles <100 μm in diameter.[4,5] Panx2 has been observed in SMCs of the pulmonary artery of mice and in the SMCs of the rat middle cerebral artery (MCA).[4,7] Although mRNA of all three pannexin isoforms was found in the rat MCA, only Panx1 and Panx2 proteins were found in ECs of these vessels.[7] As with

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any membrane-bound protein that is expressed endogenously in low levels, it is rather difficult to detect pannexin mRNA from small vessels. False-negative detection is a rather common occurrence and thus cannot be taken to mean a lack of expression. For this reason, protein detection in small vessels, in particular, is the most definitive way to detect Pannexin 1; this in conjunction with a knockout animal to validate expression. Overall, mouse and human expression appears to be the most consistent and reliable expression patterns, and thus physiologically relevant set of models, whereas the rat appears to be more random in expression.

On Panx1, a number of serine, threonine (T), and tyrosine (Y) phosphorylation sites have been predicted based on their amino acid sequences, as well as putative recognition sites for protein kinase C, protein kinase A, and Ca^{2+}/calmodulin-dependent protein kinase.[8-10] However, there is still a lack of direct biochemical evidence to link specific amino acid/kinase pairs with channel function. The current landscape of Panx1 post-translational modification by phosphorylation is dominated by the regulatory role of tyrosine phosphorylation, which plays a unique and crucial role in regulating vascular function.[11-13]

Mechanistically, a link between pannexin channel gating and tyrosine kinase phosphorylation was established using Panx1-expressing J778 macrophages and targeting the C-terminal Y308 amino acid of Panx1 in rodent hippocampal brain slices.[14,15] In the vasculature, Lohman *et al.* recently confirmed the role for Src family kinase (SFK)-dependent tyrosine phosphorylation at residue Y198 of Panx1 in response to tumor necrosis factor-α (TNFα)-receptor stimulation in the venous endothelium.[5] In this study, stimulation of ECs with TNFα resulted in an SFK-dependent increase in phosphorylation of Panx1 at Y198, which was paralleled by an increase in SFK activity.[5]

Moreover, the Panx1 Y198 site was suggested to regulate SMC contraction and vascular tone in resistance arteries.[16] Based on earlier work that characterized a novel interaction between the α1-adrenergic receptor and Panx1-mediated ATP release, Billaud *et al.* demonstrated that pharmacological and genetic inhibition of the Panx1 intracellular loop motif containing Y198 prevents the Panx1 channel activation, ATP release, and vasoconstriction initiated by α1-adrenoceptor stimulation.[16,17] In addition, this study showed that SMC-specific Panx1 deletion in mice, which exhibit blunted phenylephrine-stimulated vasoconstrictor responses, could be rescued by transfecting wild-type Panx1 plasmids directly into arterial SMCs but not by plasmids containing a mutated Y198 motif. These investigations suggest that Panx1 tyrosine phosphorylation within both ECs and SMCs could play a role in the regulation of vascular function.[5,16] However, this has yet to be proven definitely and likely is a part of a much larger post-translational modification (PTM) set of events that have yet to be uncovered.

Outside of tyrosine phosphorylation, little is known about the regulation of pannexin by serine/threonine. In one study, using pan-phosphoserine/threonine antibodies, the electrical stimulation of skeletal muscle was shown to enhance serine/threonine phosphorylation of Panx1.[18] The increase in phosphorylation was associated with ATP release and dye uptake, which was sensitive to channel blocking agents. In a second study, the Panx1 residue S206 has been put forth as a putative serine phosphorylation site by protein kinase G (PKG).[19] The inhibitory effect of the nitric oxide (NO) donor sodium nitroprusside (SNP) on Panx1 channel currents was shown to act through a cGMP-PKG dependent mechanism and mutation of the serine at residue 206 to alanine blunted the SNP-dependent inhibition of Panx1 channel currents.[19] However, this investigation did not directly demonstrate substrate specificity of PKG for S206. In the future, it will be important to determine if S206 phosphorylation by PKG negatively affects Panx1 channel activity a sPanx1-mediated ATP release has been shown to control α1-adrenoceptor-induced vasoconstriction, and PKG signaling pathways are known to cause cessation of vascular SMC contraction.[16,20]

Evidence also indicates that Panx1 channels can be regulated by S-nitrosylation. It was previously demonstrated that Panx1 channels can be activated by ischemic conditions in neurons and that inhibition of the neuronal isoform of NO synthase during oxygen/glucose deprivation blocks Panx1 channel activity in an NO-dependent and redox-sensitive manner.[21,22] Direct evidence for a potential PTM site at Panx1 residue C28 was later identified in zebrafish using targeted mutagenesis experiments on cysteine residues in the intracellular and transmembrane domains of Panx1.[23] Two later studies revealed additional residues (C40 and C346) as potential PTM sites of Panx1.[24] Mutations of these two individual residues to serines resulted in a constitutively active channel.[24] Moreover, substitution of any of the four extracellular cysteine residues in Panx1 (C66, C84, C245, and C264) resulted in complete loss of channel function.[25] Although the previous studies did not identify Panx1 S-nitrosylation in a physiological context, they did set the stage for an investigation by Lohman *et al*., which demonstrated that multisite S-nitrosylation may be important for Panx1 channel gating in the vasculature.[26] The treatment of human embryonic kidney and human amniotic epithelial cells with the NO donor S-nitrosoglutathione (GSNO) induced Panx1 S-nitrosylation; however, instead of activating Panx1 as observed in neurons, NO had an inhibitory effect on channel currents and ATP release. This inhibition could be reversed by the reducing agent dithiothreitol. Furthermore, dual mutation

of both C40 and C346 residues to alanine, but not either point mutation alone, was necessary to prevent GSNO-stimulated inhibition of Panx1 channel currents and ATP release. The results of this study highlight a potential negative regulatory mechanism of Panx1 channel gating by S-nitrosylation, which may balance vasoconstrictor responses produced by Panx1-mediated ATP release in SMCs of resistance arteries.[16]

VASCULAR PHYSIOLOGY OF PANNEXINS

The vasoconstrictor response initiated by α1-adrenoceptor activation in arterioles appears to be very complex and also involves autocrine signaling mediated by ATP release through pannexin channels. Recently, Billaud *et al.* showed that stimulation of α1-adrenoceptors leads to Panx1 channel opening, which provides the pathway for ATP release[17] [Figure 1]. The ATP released through Panx1 channels contributes, in great part, to the α1-adrenoceptor-mediated vasoconstriction through the activation of P2Y receptors.[16,17,27] Interestingly, this complementary vasoconstrictor mechanism is only coupled to α1-adrenoceptor activation while the response to serotonin or endothelin-1 is not affected by typical Panx1 channel blockers such as the Panx1 mimetic peptide ^{10}Panx and probenecid, which was confirmed in the SMC Panx1-deficient mice.[16] This observation has now been confirmed independently pharmacologically[28] and genetically[29] in mouse mesenteric arterioles (as well as humans [data not shown]) highlighting the importance of the Panx1-α1-adrenerigc interaction, especially for translational outcomes.

ECs play a central role in the regulation of vasomotor tone primarily by the production of Ca^{2+}-dependent vasodilator signals such as NO, prostaglandins, and endothelium-derived hyperpolarization (EDH).[30-33] The global deletion of Panx1 resulted in a selective reduction of the SK_{Ca} and IK_{Ca}-initiated EDH-mediated vasodilation, without affecting the NO-dependent vasodilator component of the response to acetylcholine, although both signals (EDH and NO) are Ca^{2+} dependent.[34] Ablation of Panx1 in these animals did not evoke a compensatory change in the expression of Panx2 or Panx3 in the saphenous artery; thus, the mechanism by which Panx1-driven ATP release activates selectively SK_{Ca} and IK_{Ca} in ECs will have to be addressed in further investigations.[34]

VASCULUAR PHYSIOLOGY OF INFLAMMATORY RESPONSES

The pro-inflammatory TNFα-induced ATP release was shown to be Panx1-dependent. Lohman *et al.* recently identified an important mechanism of regulation of endothelial Panx1 channels in TNFα-induced inflammation *ex vivo*, using dissected mesenteric veins, and *in vitro*, using isolated human primary venous ECs.[5] Application of TNFα induced, within minutes, ATP release into the lumen of cannulated veins or the media of cultured cells. Importantly, this effect was only seen in veins or venous ECs but not in arteries or arterial ECs. This ATP release was Panx1 channel-mediated as shown by the prevention of ATP release with pharmacological inhibitors, such as carbenoxolone (CBX) or ^{10}Panx, and with siRNA-mediated knockdown of Panx1. siRNA knockdown of Cx43, conversely, had no effect. Furthermore, cannulated veins from EC-specific Panx1-deficient mice failed to release ATP following TNFα stimulation. TNFα induced SFK-dependent phosphorylation at tyrosine Y198 in the intracellular loop of Panx1, leading to the opening of the channel. Intravital microscopy demonstrated that TNFα-induced leukocyte adhesion and emigration was blunted in EC-specific Panx1-deficient mice.[5] In a similar study, thrombin induced a robust, Ca^{2+}-dependent ATP release through Panx1 channels in HUVECs.[35] These results highlight Panx1 as a key protein for the cross talk between cytokine and purinergic signaling in early inflammation.

A functional role of Panx1 was also suggested in the signaling between the nervous and vascular system under pro-inflammatory conditions. Stimulation of primary arterial endothelial mesenteric cells and intact mesenteric arteries with the neurotransmitter calcitonin gene-related peptide (CGRP) leads to Panx1 channel opening as shown by ethidium uptake, which was prevented by the specific inhibition of CGRP receptors with the peptide $CGRP_{8-37}$ or the blockade of Panx1 channels with probenecid.

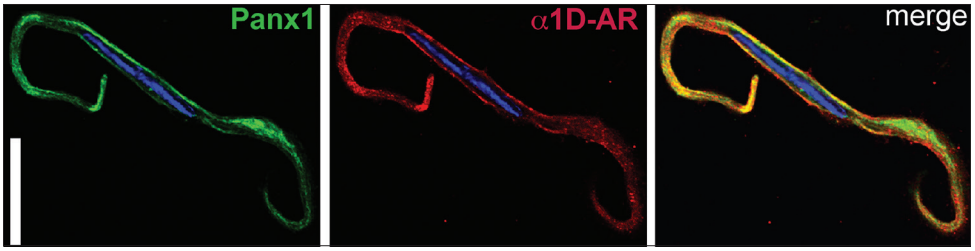

Figure 1: Freshly isolated smooth muscle cell (<1 h) after being enzymatically digested from a mouse mesenteric arteriole. Note the expression of Panx1 and the alpha1D-adrenergic receptor in close proximity (yellow). Scale bar is 10 μm

Furthermore, stimulation of perivascular sensory nerves with capsaicin led to CGRP release and downregulation of endothelial NO synthase (eNOS) expression, linking pro-inflammatory-induced neurotransmitter release to the development of endothelial dysfunction during inflammation through decreased eNOS protein levels.[36]

PANNEXINS IN CIRCULATING CELLS

Long viewed simply as carriers of oxygen, in the past two decades, red blood cells (RBCs) have received renewed attention for their potential "erythrocrine" function; that is, the ability to influence their own distribution across the vasculature by participating in signaling pathways that result in the modulation of vascular tone, thus controlling blood flow to tissues with relatively greater or lesser need of oxygenation.[37-39] Ellsworth *et al.* proposed in 1995 a pathway involving RBC release of ATP into the vessel lumen based on the knowledge that RBCs release ATP in response to hypoxic conditions and that ATP application results in significant increases in RBC supply rate to both arterioles and venules at the end of a capillary network.[37,40] ATP released by RBCs is thought to bind to G protein-coupled P2Y receptors on the endothelium, initiating a signaling cascade to produce and release peripheral vasodilatory signaling molecules such as NO and prostacyclin.[37] Upstream transmission of this vasodilator response allows blood flow to be modulated to match local oxygen demand.[41] ATP is released when human RBCs are exposed to O_2 tensions equivalent to 50% hemoglobin oxygen saturation.[42] Further, supporting the central role of RBCs in this process, perfusion of blood vessels with RBCs was shown to be necessary to elicit vessel dilation in response to hypoxia.[43] This phenomenon is also supported by exercise data showing that changes in the levels of circulating ATP in blood plasma are proportional to changes in the oxygenation state of hemoglobin in RBCs.[38]

A mechanism for ATP release was provided by the detection of functional Panx1 channels in RBCs and by the lack of Cx43 and vesicular release of ATP in RBCs under physiological conditions.[44,45] Inhibition of RBC ATP release with the pannexin inhibitors CBX, probenecid, and ^{10}Panx later clarified that Panx1 could be responsible for ATP release from human RBCs in response to hypoxia though not for a separate mechanism of ATP release in response to activation of the prostacyclin receptor.[46] In addition, mechanical deformation of RBCs has been found to induce ATP release through a pathway similar to that of hypoxia-induced ATP release, with impairment of RBC deformability also impairing hypoxic ATP release.[42,47] The exact mechanism of Panx1 opening in response to hypoxia or RBC deformation remains unsolved, but a variety of data suggests that Panx1 lies downstream of the mechanosensitive G-protein-coupled receptor G_i, (e.g., P2Y receptors), adenylyl cyclase, and cyclic adenosine monophosphate synthesis.[42,46,48] However, RBCs are still able to release ATP in response to direct G_i stimulation when RBC deformation is prevented. These observations suggest that hypoxia and deformation of RBCs are linked, possibly by G_i to initiate the signaling cascade culminating in the Panx1-dependent release of ATP into the vessel lumen.[47] The resulting NO that serves as a vasodilator may, in turn, inhibit ATP release in a negative feedback loop.[42] While the mechanism has not been fully explained, this negative feedback is thought to occur through NO-derived inactivation of G_i.[48] However, the recent discovery that Panx1 can be inhibited directly by NO through S-nitrosylation on amino acid residues C40 and C346 offers an alternative explanation that must be investigated.[26]

Platelets are anuclear cells found in blood that, in the presence of endothelial damage, bind exposed collagen on the basement membrane of the vessel wall. Further, they are activated to release α-granules that contain a variety of growth and clotting factors and dense granules that contain adenosine diphosphate, ATP, Ca^{2+}, serotonin, and histamine; signals that promote further platelet aggregation.[49] Certain pathologic conditions result in elevated platelet activity outside of normal hemostasis, including chronic infectious or inflammatory conditions such as atherosclerosis.[49] When an atherosclerotic plaque ruptures, platelets erroneously become activated, often leading to thrombosis and possible vessel occlusion.[50] Interrupting pathogenic platelet activation and aggregation is, therefore, an important strategy to prevent and combat thrombosis.

Panx1 has also been identified on the surface of human platelets but not Panx2 or Panx3.[51,52] Panx1 inhibition or deletion in platelets resulted in impaired collagen-induced aggregation, ATP release, and Ca^{2+} influx.[51,52] Molica *et al.* reported that, in platelets, Panx1 co-immunoprecipitates with $P2X_1$, an ATP-gated channel involved in thrombosis.[51] Inhibition of $P2X_1$ with NF449 decreased collagen-induced platelet aggregation, which was not restored by potassium-stimulated opening of Panx1 channels.[51] Taylor *et al.* found that the Panx1 inhibitors probenecid and CBX, impaired collagen, thrombin, and thromboxane A_2 analog-induced Ca^{2+} influx, possibly through $P2X_1$ signaling.[52,53] Together, these data suggest that $P2X_1$ signaling occurs downstream of Panx1.[51-53] Platelets from patients homozygous for the Panx1-400C polymorphism, which results in a change from glutamine to histamine at residue 5 in the Panx1 amino-terminus, release elevated amounts of ATP at rest when stimulated with K^+ and demonstrate increased collagen-induced platelet

reactivity compared to platelets with the Panx1-400A allele coding for glutamine.[51] In addition, a higher frequency of the Panx1-400C allele was found in cardiovascular patients with hyperreactive platelets compared to those with hyporeactive platelets.[51] Further evaluation of pannexin-dependent signaling in platelets may provide a new therapeutic target for cardiovascular patients.

CONCLUSION

Although some work on the Panx1 isoform has been elucidated, there are many answered and fascinating questions yet to be answered. What is the role of other pannexin isoforms? What are other factors that may gate pannexin channel opening and closing? Are the pannexin channels in complex with other purinergic signaling molecules. This is an exciting time to be a "pannexin-ologist," especially in the vasculature.

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Conflicts of interest

There are no conflicts of interest.

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