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Connexin 43 is an emerging therapeutic target in ischemia/reperfusion injury, cardioprotection and neuroprotection

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Abstract

Connexins are widely distributed proteins in the body that are crucially important for heart and brain function. Six connexin subunits form a connexon or hemichannel in the plasma membrane. Interactions between two hemichannels in a head-to-head arrangement result in the formation of a gap junction channel. Gap junctions are necessary to coordinate cell function by passing electrical current flow between heart and nerve cells or by allowing exchange of chemical signals and energy substrates. Apart from its localisation at the sarcolemma of cardiomyocytes and brain cells, connexins are also found in mitochondria where they are involved in the regulation of mitochondrial matrix ion fluxes and respiration. Connexin expression is affected by age and gender as well as several pathophysiological alterations such as hypertension, hypertrophy, diabetes, hypercholesterolemia, ischemia, post-myocardial infarction remodelling or heart failure, and post-translationally connexins are modified by phosphorylation/de-phosphorylation and nitros(yl)ation which can modulate channel activity. Using knockout/knockin technology as well as pharmacological approaches, one of the connexins, namely connexin 43, has been identified to be important for cardiac and brain ischemia/reperfusion injury as well as protection from it. Therefore, the current review will focus on the importance of connexin 43 for irreversible injury

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of heart and brain tissue following ischemia/reperfusion and will highlight the importance of connexin 43 as an emerging therapeutic target in cardio- and neuroprotection.

1. General introduction to connexins

Connexins (Cx) are widely distributed proteins in the body that are crucially important for heart and brain function. Connexins are integral membrane proteins which span the plasma membrane four times with N- and C-terminal ends facing the cytosol (Figure 1). Six Cx monomers form a connexon or hemichannel in the plasma membrane. Interactions between the extracellular loops (EL) of two hemichannels in a head-to-head arrangement result in the formation of a gap junction channel (reviewed in Desplantez et al., 2007; Saez et al., 2003). In this process, the two closed hemichannels open, thereby forming a channel that directly connects the cytoplasm of the two adjacent cells allowing the exchange of ions and small molecular weight substances up to ~2 kDa. Gap junctions are assemblies of closely packed gap junction channels that often appear as gap junction plaques containing thousands of gap junction channels. Gap junction plaques are typically observed in the heart at the intercalated disks of adjacent cardiomyocytes (Revel & Karnovsky, 1967; Severs, 1990) where they facilitate electrical current flow that coordinates cardiomyocyte contraction to sustain its pump function (Severs et al., 2004). In the brain, gap junctions communicate electrical signals between neurons (Pereda, 2014) and pass chemical signals and metabolites (glucose, lactate) between glial cells to support the function of the neuronal, glial and vascular cell ensemble in the neurovascular unit (Giaume et al., 2010).

Hemichannels are precursors of gap junctions but they also exist as free, non-junctional channels in the plasma membrane. They are normally closed but may open in response to various triggers including cell depolarization, decreased extracellular calcium ion (Ca^{2+}) concentration, increased intracellular Ca^{2+} concentration and alterations in the phosphorylation or redox status (D'Hondt et al., 2014; Giaume et al., 2013; Orellana et al., 2013; Saez & Leybaert, 2014). In the brain, glial plasma membrane hemichannels may function as gliotransmitter release pores (Cheung et al., 2014; Montero & Orellana, 2015; Orellana & Stehberg, 2014). Most of the evidence currently available, however, implicates plasma membrane hemichannels as pathological rather than physiological entities, contributing to cell swelling and cell death. In heart and brain cells, excessive hemichannel opening allows the entry of sodium (Na^+) and Ca^{2+} and the escape of potassium (K^+), adenosine triphosphate (ATP) and other small metabolites, leading to osmotic shifts, energy depletion, Ca^{2+} overload and cell death promotion (John et al., 1999; Kondo et al., 2000; Bargiotas Monyer, & Schwaninger, 2009; Contreras, et al., 2004; Davidson et al., 2013; Davidson et al., 2014; Decrock et al., 2009; Li et al., 2001; Orellana et al., 2014; Wang et al., 2013).

Mutations of the Cx protein may lead to congenital diseases; the most frequent one is hearing loss linking to Cx26, Cx30 and other Cxs (Kelly et al., 2014). In the heart atrial fibrillation may result from mutations in Cx40 (Molica et al., 2014). Cxs also play prominent roles in acquired diseases. Under pathological conditions, gap junctions may expand cell injury/cell death to surrounding healthy cells causing bystander (Lin et al., 1998) or “spreading of injury” (Garcia-Dorado et al., 2004) effects. Conversely, gap junctions may

also act in a protective manner by supplying essential nutrients and metabolites, thereby preventing injury or death of healthy neighboring cells (Decrock et al., 2009). In cardiac infarction, ischemia triggers a decrease in gap junctional communication as a result of acidosis, increased intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and altered phosphorylation and nitrosylation levels (Fig. 2). This along with alterations in other ion channels, leads to slowed electrical impulse conduction and increased risk for arrhythmias (Cascio et al., 2005; Dhein, 2006; Dhein et al., 2014). In brain ischemia, astrocytic gap junctional coupling decreases, and like in heart, closure is incomplete (Cotrina et al., 1998; Eugenin et al., 2012). Decreased coupling hampers the spatial buffering of potassium and glutamate, and limits sharing of energy substrates from the astrocytic syncytium to neurons (Contreras et al., 2004; Rossi et al., 2007). Unfortunately, the remaining coupling may be sufficient to propagate bystander injury and cell death between astrocytes and between gap junction-coupled neurons (Belousov & Fontes, 2013).

In physiology as well as in pathology, Cx functions are not always linked to their roles as channels (reviewed in Giepmans, 2004; Vinken et al., 2012)). Many Cxs can be anchored to scaffolding proteins via CT interaction. For example, the CT of connexin 43 (Cx43) can interact with the PDZ-domain of zona occludens protein-1 (ZO-1), linking it to the cytoskeleton. CT-ZO-1 interaction may compete with the binding of transcription factors to ZO-1 (for example, ZO-1-associated nucleic acid binding proteins (ZONAB), a transcription repressor) and in this way influence gene expression (Balda & Matter, 2000). Interaction of the CT with β -catenin may influence Wnt signaling while interactions between CT and c-Src (Herrero-Gonzalez et al., 2010) inhibits c-Src-related oncogenic activity and key steps of cell cycle regulation (Zhang et al., 2003). Finally, the Cx43 CT can potentially localize to the nucleus and thereby inhibit cell growth (Dang et al., 2003; Vinken et al., 2012).

The most abundant Cx in brain and heart is Cx43. In the brain, Cx43 is most prominently expressed in astrocytes but is also present in microglial cells (Giaume & Theis, 2010). Astrocytes additionally express Cx30 and microglial cells express Cx32 and Cx36. Oligodendrocytes express Cx32, Cx47 and Cx29, and neurons mainly Cx36. Brain capillary endothelial cells that form the blood-brain barrier mainly express Cx37 and Cx40, with some low Cx43 signal that is increased with inflammation (Cronin et al., 2008; Danesh-Meyer et al., 2012). Neurons do not express Cx43 but they do express Cx36 and Cx45.

In the heart, Cx43 is most prominently expressed in cardiomyocytes of the ventricles, with some signals obtained also in the atria and endothelial cells (Severs et al., 2008). The atria mainly contain Cx40, which is also expressed together with Cx37 in coronary endothelial cells. Apart from Cx43, the special conduction system in the ventricles contains Cx45. In the atrioventricular and sinoatrial node of mice, Cx30.2 is expressed while its orthologous protein Cx31.9 is not detectable in the human cardiac conduction system (Kreuzberg et al., 2008).

Using knockout/knockin and pharmacological approaches Cx43 has been shown to play important roles during cardiac and brain ischemia/reperfusion injury as well as protection from it. Therefore, the current review will summarize the existing evidence for the role of

Cx43 in irreversible injury of heart and brain tissue following ischemia/reperfusion highlights Cx43 as an emerging drug target for cardio- and neuroprotection.

2. Regulation of Cx43 in heart and CNS

2.1. Posttranslational modification

With an increasing number of new phosphorylation specific antibodies and constructs, research related to Cx43 regulation has gone past a simple correlation of unspecified phosphorylation-related migration shifts on Western blots to an approach whereby Cx43 phosphorylation can be mechanistically linked to changes in Cx43-interacting protein binding, gap junctional communication, hemichannel function, kinase activity and the underlying signalling pathways affecting cell biological function (Chen et al. 2013; Cooper & Lampe, 2002; Dunn et al., 2012; Dyce et al., 2012; Ek-Vitorin et al., 2006; Johnstone et al., 2009; Johnstone et al., 2012; Lampe et al., 1998b; Lampe et al., 2006; Marquez-Rosado et al., 2012; Solan & Lampe, 2008; Solan et al., 2007; TenBroek et al., 2001).

Cx43, like Cx31, 32, 37, 40, 43, 45, 46, and 50 is a phosphoprotein with clear evidence for phosphorylation at more than 12 serine (S) and tyrosine (Y) sites in the CT region of the protein via at least 6 kinases (Axelsen et al., 2006; Dunn et al., 2012; Ek-Vitorin et al., 2006; Johnstone et al., 2009; Johnstone et al., 2012; Lampe, 1994; Lampe et al., 1998a; Lampe et al., 2006; Marquez-Rosado et al., 2012; Richards et al., 2004; Saez et al., 1998; Solan & Lampe, 2008; Solan et al., 2007; Stagg & Fletcher, 1990; TenBroek et al., 2001). Also like most other Cx, Cx43 has a short half-life reported to be ~2 hours in cell lines and cardiac tissue (Beardslee et al., 1998; Crow et al., 1990; Darrow et al., 1995; Hertlein et al., 1998; Laird et al., 1991; Lampe, 1994; Musil et al., 1990; Musil & Goodenough, 1991), and phosphorylation status has been linked to differences in Cx43 localization and half-life (Axelsen et al., 2006; Dunn & Lampe, 2014; Lampe, 1994; Lampe et al., 1998a; Marquez-Rosado et al., 2012; Richards et al., 2004; Solan et al., 2007). Truncated Cx43 that lacks the CT portion (Cx43K258 stop) can form gap junctions but their channels have different permeability/electrophysiological properties (Dunham et al., 1992; Fishman et al., 1991; Moreno et al., 2002). Furthermore, truncated Cx43 has a prolonged half-life and a knockin mouse expressing truncated Cx43 (Cx43K258stop) died shortly after birth (Maass et al., 2004).

Cx43 phosphorylation at S364 (TenBroek et al., 2001) and S365 (Solan et al., 2007) increases in response to stimuli that enhance gap junction assembly, and phosphorylation at S325/328/330 via casein kinase 1 (CK1) regulates assembly into gap junction channels (Cooper & Lampe, 2002; Lampe et al., 2006).

Cx43 localization and phosphorylation are dramatically modulated during ischemia and injury (Axelsen et al., 2006) most likely depending on the cellular ATP content (Turner et al., 2004).

In isolated rat (Matsushita et al., 2006) and rabbit (Tansey et al., 2006) hearts, prolonged ischemia/hypoxia (>15 minutes) induces sarcolemmal redistribution of Cx43 with a reduction of gap junctional Cx43 [and N-cadherin (Tansey et al., 2006)] and increased

expression at the lateral surface of cardiomyocytes (Beardslee et al., 2000). These changes are concomitant with changes in Cx43 phosphorylation: ischemia results in a 8-fold loss of pS365 (Solan et al., 2007) and S325/328/330 (Lampe et al., 2006), and a 5-fold and 3.5-fold increase at S368 (Ek-Vitorin et al., 2006) and S373, respectively (Dunn & Lampe, 2014). The dephosphorylation of S365 in response to ischemia occurs rapidly (5 min) (Sosinsky et al., 2007) followed by increases in S368 phosphorylation, consistent with a “gatekeeper” concept where S365 phosphorylation prevents S368 phosphorylation leading to an inverse relationship in vivo (Solan et al., 2007). Thus, the role of Cx43 phosphorylation in regulating cardiac injury in response to hypoxia appears to be multifold. Proteasomal inhibition increases and stabilizes phosphorylated Cx43 present in gap junctions via increased Akt activity (Dunn et al., 2012), and Akt phosphorylation of Cx43 at S373 (pS373) eliminates ZO-1 interaction and causes a dramatic increase in gap junction size that can be mimicked by expression of Cx43 with a S373D mutation (Dunn & Lampe, 2014). Cx43 is sequentially phosphorylated (Figure 1) by Akt (Dunn & Lampe, 2014), c-Src (Solan & Lampe, 2008), mitogen activated protein kinases (MAPK) (Johnstone et al., 2012) and protein kinase (PKC) (Lampe, 1994; Richards et al., 2004; Solan et al., 2003) in response to growth factors, hypoxia and other stimuli which induce acute gap junction turnover. Gap junction size is negatively regulated by Cx43 interaction with ZO-1 in experiments where the endogenous ZO-1:Cx43 interaction was outcompeted with a Cx43 CT peptide mimetic resulting in a dramatic increase in gap junction size (Hunter et al., 2005). During ischemia, S373 phosphorylation is coincident with an increase in gap junction size and precedes internalization (Dunn & Lampe, 2014). Internalized Cx43 in isolated mice hearts following 30 min global ischemia shows increased phosphorylation at Ser373 and Ser368 which enables the binding of the 14-3-3 protein leading to ubiquitination of Cx43 (Smyth et al., 2014), a process which can be attenuated by protein kinase C inhibition (Liao et al., 2013).

A gap junction can be internalized in its entirety via formation of a double membrane structure termed an annular junction (Archard & Denys, 1979; Fong et al., 2012; Johnson et al., 2013; Jordan et al., 2001; Laird, 2006; Leithe et al., 2006; Nickel et al., 2013; Piehl et al., 2007; Severs et al., 1989) or via loss of extracellular Cx interactions followed by endocytosis of the remaining connexon from a single membrane. During internalization, Cx43 can be phosphorylated by c-Src and inhibition of c-Src activity via protein phosphatase 2 blocks growth factor-induced gap junction turnover (Gilleron et al., 2008; Spinella et al., 2003). Glycyrrhetic acid-related gap junction inhibitors remodel gap junctions into a looser packing arrangement (Goldberg et al., 1996) in a process that involves c-Src binding (Chung et al., 2007) and leads to disruption of Cx43-ZO-1 interaction (Gilleron et al., 2008). C-Src can directly interact with ZO-1 and compete for binding to the CT region of Cx43 (Gilleron et al., 2008; Kieken et al., 2009; Sorgen et al., 2004). Clearly, c-Src plays a role in gap junction turnover, but whether c-Src phosphorylation of Cx43 plays a direct role is not known. C-Src phosphorylation of the N-methyl-d-aspartate (NMDA) receptor, GluN3A (Chowdhury et al., 2013), triggers its endocytosis. Whether c-Src phosphorylation can direct the endocytic route of internalization through annular junction formation or by “unzippering” gap junctions via loss of extracellular interactions followed by endocytosis is unknown. The formation of double membrane endocytic vesicles (i.e., annular junctions) appears to be fairly specific to gap junctions though there are a few

reports of “trans-endocytosis” occurring in dendritic cells (Spacek & Harris, 2004) and in response to receptor ligand complex formation during neural (Marston et al., 2003) and *Drosophila* development (Klueg et al., 1998). Cx43-containing annular junctions have been well-documented (Archard & Denys, 1979; Fong et al., 2012; Johnson et al., 2013; Jordan et al., 2001; Laird, 2006; Leithe et al., 2006; Nickel et al., 2013; Piehl et al., 2007; Severs et al., 1989) but the role Cx43 phosphorylation plays is still unclear.

Knockin of Cx43 phosphoserine-site mutants (S to A, resulting in a non-phosphorylation mutant version of Cx43 at that site) into mice are of particular interest to determine the functional necessity for Cx43 phosphorylation *in vivo*. Three knockin mice lines with Cx43 mutations at sites phosphorylated by MAPK, CK1 and PKC have been generated, all knocked-in under the endogenous Cx43 promoter (referred to as Cx43^{PKC}, Cx43^{CK1} and Cx43^{MAPK} knockin mouse lines) (Huang et al., 2011). All 3 knockin mice lines show different changes in Cx43 phosphorylation, different migration in SDS-PAGE, and varying gap junction stability (e.g., two distinctly derived lines of the Cx43^{PKC} KI mouse show 60% reduced Cx43 levels in heart). The Cx43^{CK1} mice where the S325/328/330 serines are converted to glutamates were resistant to pathological remodeling and to the induction of ventricular arrhythmias while the line with conversion to alanine were prone to it (Remo et al., 2011). The Cx43^{MAPK} mice show dramatically reduced vascular smooth muscle cell proliferation and neointima formation during arteriole injury (Johnstone et al., 2012).

These results prove that Cx43 phosphorylation plays a key role in modulation of the response to injury in heart and brain *in vivo* and that these findings might translate into putative therapeutic applications in the future.

2.2. Expression of Cx43

Estrogen (Yu et al., 1994) and triiodothyronine (Stock and Sies, 2000) directly bind to the promoter region of the Cx43 gene thereby increasing its expression. Other transcription factors involved in the regulation of Cx43 expression in cardiomyocytes are TBX18 (family member of T-box transcription factor suppressing Cx43, Kapoor et al., 2011), MSX1/2 (Msh homeobox suppressing Cx43, (Boogerd et al., 2008)), AP1 and CREB (cAMP responsive element binding protein stimulating Cx43, (Salameh et al., 2009)) or NKx2.5 (suppressing Cx43, (Kasahara et al., 2003)). AP1 and CREB are translocated to the nucleus secondary to the activation of PKA or MAPK, such as p38 or extracellular regulated kinase (Erk) 1/2 (Salameh et al., 2009).

Cyclic stretch increases N-cadherin expression at the longitudinal sites of cardiomyocytes, a process requiring rho/rac (Matsuda et al., 2006). Loss of N-cadherin reduces Cx43 expression at gap junctions (Li et al., 2008; Li et al., 2005; Palatinus et al., 2011; Zhu et al., 2010). As cyclic stretch increases N-cadherin in cardiomyocytes, it also increases total Cx43 expression and polarization of Cx43 to the longitudinal sites (Salameh et al., 2012). The increases in Cx43 expression and localisation to gap junctions in stretched cardiomyocytes involve angiotensin II, Akt, Erk1/2 and glycogen synthase kinase (GSK) 3 β (Salameh et al., 2012; Salameh et al., 2010a; Salameh et al., 2010b; Shyu et al., 2001). Angiotensin II also increases Cx43 expression (Dodge et al., 1998; Polontchouk et al., 2002) and N-cadherin expression in cardiomyocytes (Adam et al., 2010).

In addition to angiotensin II, other stimuli can also increase Cx43 expression in cardiomyocytes including norepinephrine (Salameh et al., 2006;Salameh et al., 2008), endothelin 1 (Polontchouk et al., 2002), low concentration of aldosterone (Suzuki et al., 2009) and tumor necrosis factor alpha (Salameh et al., 2004). However, long-term treatment with stimuli inducing cardiomyocyte hypertrophy might also activate signaling cascades which suppress Cx43 expression; indeed, norepinephrine caused by activation of c-Jun N-terminal kinase (JNK)1/2 attenuates Cx43 expression over time, an effect that also involves the sodium-proton and sodium-calcium exchanger (Stanbouly et al., 2008). Over-expression of active JNK decreases Cx43 expression at gap junctions (Petrich et al., 2002) and may activate Cx43 degradation through ubiquitination and proteosomal degradation (Mollerup et al., 2011). Similar to norepinephrine, endothelin-1 increases cardiomyocyte size more than Cx43 expression, leading to a relative reduction in sarcolemmal Cx43 expression and cardiomyocyte coupling (McSpadden et al., 2009). Cx43 in cardiomyocytes is not only involved in cellular coupling via gap junctions but through formation of hemichannels that play a role in basal calcium signaling (Li et al., 2012). While signaling in cardiomyocytes is important, the interaction of cardiomyocytes with other cell types may modify these responses. Prolonged stimulation of cardiomyocytes with isoprenaline increases Cx43 expression through activation of Erk1/2 (Salameh et al., 2013;Salameh et al., 2009); however, in co-cultures of cardiomyocytes and fibroblasts, paracrine factors released by fibroblasts suppress the isoprenaline-induced increase in Cx43 expression in cardiomyocytes (Pedrotty et al., 2009;Salameh et al., 2013). Such suppression can partially be reversed by blocking angiotensin II or PKC (Pedrotty et al., 2009), and in cardiac tissue from mice, inhibition of the renin angiotensin system increases Cx43 expression (Irvanian et al., 2011).

2.3. Ischemia/hypoxia-induced alterations of Cx43 expression

Hypoxia of cardiomyocytes changes the Cx43 expression and phosphorylation time-dependently. During short periods of hypoxia (up to 15 minutes) Cx43 content remains unchanged (Matsumura et al., 2006;Zeevi-Levin et al., 2005). With prolonged hypoxia (several hours), downregulation of Cx43 at gap junctions occurs (Danon et al., 2010), Cx43 is internalized (Sato et al., 2009) and subsequently the total cellular Cx43 content decreases (Zeevi-Levin et al., 2005).

Metabolic inhibition activates c-Src kinase, an effect inhibited by 17 β -estradiol (Chung et al., 2009). Furthermore, 17 β -estradiol treatment preserves Cx43 phosphorylation at PKC epitopes during metabolic inhibition (Chung et al., 2004) thereby contributing to maintained localization of Cx43 to gap junctions.

In cardiomyocytes, the muscarinic M3 receptor is associated with Cx43 (Yue et al., 2006) and the hypoxia-induced decrease in total Cx43 expression is attenuated by acetylcholine (Zhang et al., 2006). Similarly, proteasome inhibitors attenuate the hypoxia-induced decline in Cx43 expression (Laing and Beyer, 1995;Zhang et al., 2006).

In summary, stimulation of Cx43 expression in Cx43 deficient pathologies may be a therapeutic option to reverse loss of Cx43.

3. Pharmacological modulation of Cx43-formed channels

The available arsenal of pharmacological agents modulating (mostly inhibiting) gap junctions and hemichannels is extensive and contains chemically diverse compounds. A large group is formed by small molecule inhibitor substances; most of these have targets other than Cx and are therefore non-selective. This group (reviewed in (Bodendiek & Raman, 2010)) includes glycyrrhetic acid and its derivative carbenoxolone (steroid-like structures from *Glycyrrhiza* also exhibit anti-inflammatory and mineralocorticoid effects), long-chain alcohols like heptanol and octanol (having general biophysical effects at the level of the plasma membrane), halothane (an inhalational general anesthetic), fatty acids like arachidonic acid (a poly-unsaturated fatty acid present in plasma membrane phospholipids and involved in phospholipase C (PLC) and inflammatory signaling) and oleic acid (a mono-unsaturated fatty acid with various biological effects, abundantly present in olive oil), fatty acid amides like oleamide and anandamide (targeting cannabinoid receptors), fenamates like flufenamic acid, niflumic acid and meclofenamic acid (non-steroid anti-inflammatory molecules), and quinine, its stereo-isomer quinidine and its derivative mefloquine (antimalarial drugs). Several other compounds that inhibit gap junctions are best known for their other actions, like 2-aminoethoxydiphenyl borate (2-APB, a blocker of inositol trisphosphate (IP₃) receptors and store-operated Ca²⁺ entry (Bootman et al., 2002)), polyamines like spermine and spermidine (acting on NMDA channels and several other targets), 5-Nitro-2-(3-phenyl-propylamino)benzoic acid (NPPB, a chloride channel blocker), disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS, an anion transport blocker), and certain triphenylmethanes, triphenylethanes, triarylmethanes and cyclodextrins. Importantly, most of these substances have been characterized for their gap junction-inhibiting effects but they invariably also inhibit hemichannels. The mode of action of arachidonic acid is different because it inhibits gap junctions but promotes hemichannel opening (Contreras et al., 2002; De Vuyst et al., 2007; De Vuyst et al., 2009). Furthermore, quinine has been reported to activate hemichannels as well (based on ATP release measurements (Stout et al., 2002)). Many of the above listed compounds additionally block channels composed of pannexins, including carbenoxolone, flufenamic acid (Bruzzone et al., 2005), NPPB and DIDS. Of note, low concentrations of carbenoxolone (5 μM) preferentially inhibit pannexin channels while higher concentrations (50 μM) are needed to also inhibit connexin channels (Schalper et al., 2008).

Because of the lack of specificity of small molecule inhibitors of Cx channels, substantial interest has been directed to peptides as agents with better selectivity potential. AAP10 peptide is one of the first peptides reported to have connexin modulating properties, and it increased gap junctional coupling. Later, peptides identical to certain Cx domains called Cx mimetic peptides, were introduced as agents with better specificity towards Cx channels.

AAP10 is a 6 amino acid peptide that was found to promote gap junctional coupling (Grover & Dhein, 1998, 2001; reviewed in Dhein et al., 2010; De Vuyst et al., 2011). It binds with nanomolar affinity to a yet unidentified membrane G-protein coupled receptor (typical active AAP10 concentrations are 10-50 nM), thereby resulting in the activation of certain protein kinases. The junctional coupling-promotive effect has been demonstrated to be mediated by activation of PKCα. There is some selectivity in the effect on gap junctions,

with Cx43 and Cx45 junctional coupling being promoted and Cx40 not affected. Rotigaptide (ZP123) is a more stable version of AAP10 composed of D-amino acids concatenated in reverse order, resulting in higher stability after administration to animals. These peptides have been shown to be effective against ischemia-induced ventricular fibrillation and possibly also atrial fibrillation (see Dhein et al., 2010). Gap-134 is a small molecule version designed based on insights obtained from the pharmacophore of AAP10. This substance has been tested in phase I clinical trials on healthy volunteers and was demonstrated to be safe (NCT00783341, 2009). Conclusive information on phase II clinical trials is lacking. Of note, AAP10 promotes hemichannel opening as judged from ATP release studies performed in cardiomyocytes exposed to ischemia-mimicking conditions (Clarke et al., 2009). Uncontrolled opening of Cx43 hemichannels can mediate Na^+ or Ca^{2+} entry and loss of intracellular K^+ , so any potential side effects of these compounds will have to be carefully followed.

Cx mimetic peptides were introduced over 20 years ago with the aim of finding sequences that had specific physiological effects (mainly channel block) on Cx channels. The first Cx mimetic peptides described were identical to sequences on extracellular loop (EL) 1 or 2. Peptide B/1 (VCYDHFPPISHVR) on EL1 and peptide5 (SRPTEKTVFTV) on EL2 of Cx32 were most effective in delaying synchrony in spontaneously beating chick cardiomyocytes in a myoball assay (Warner et al., 1995). These two peptides contain the VCYD and SHVR sequences on EL1, and the SRPTEK sequence on EL2 that are conserved between Cx32, Cx42, Cx43 and Cx45. For Cx43, the corresponding sequences are VCYDKSFPISHVR, called Gap26, and SRPTEKTIFII, called Gap27 (Figure 1). Because these sequences are very well conserved between different Cx, it comes to no surprise that Cx43-based mimetic peptides also inhibit gap junctions composed of other Cx. For example, the Cx43 sequence of Gap27 (sometimes denoted as $^{43}\text{Gap27}$) also inhibits gap junctions composed of Cx37 (Chaytor et al., 2001). However, it has not been systematically investigated whether Cx other than Cx37 and Cx43 are also targeted by $^{43}\text{Gap27}$. The Gap26/Gap27 sequences are not found in other proteins, which may be key for their specificity for the Cx family rather than non-Cx targets. However, 20 years after the discovery that Gap-peptides can block gap junctions, it still remains unknown which domains on the extracellular loops they interact with so the actual mechanism of how Gap26/27 peptides inhibit gap junctions is still elusive. They do not disrupt existing gap junctions but rather act by preventing the formation of new gap junctions (Evans & Boitano, 2001). The peptides thus probably interact with the ELs on free hemichannels and thereby hinder/prevent the docking and assembly of two hemichannels into new gap junction channels. Gap26/27 also inhibit conductance of hemichannels (Braet, Vandamme, Martin, Evans, & Leybaert, 2003). In fact, inhibition of hemichannels occurs faster, within minutes, than inhibition of gap junctions which takes something in the range of hours, at least in cell culture assays (Decrock et al., 2009). In terms of diffusion, minutes is very long suggesting that the interaction site is poorly accessible (Wang et al., 2012). Single-channel patch clamp studies of Cx43 hemichannel block indicate a half-maximal inhibitory concentration of 81 μM for Gap26 and 161 μM for Gap27. However, inhibition obtained with Gap27 is stronger as compared to Gap26. Single-channel analysis further demonstrated that Gap26/27 do not cause hemichannel pore block (provided their concentration is below 1 mM) and do not influence the unitary hemichannel

conductance of ~220 pS (Wang et al., 2012). Instead, Gap26/27 appear to act on channel gating as they reduce the open probability and increase the voltage threshold for electrical activation. Hemichannels can also be activated by chemical signals, for example by an increase in the intracellular Ca^{2+} concentration. It is not entirely clear how Gap26/27 inhibit chemically-triggered hemichannel opening but a likely scenario is that they interact with a binding site outside the permeation pathway of the closed hemichannel and thereby lock the channel in a stabilized closed state (Wang et al., 2013).

3.1. Blocking hemichannels without blocking gap junctions

As mentioned earlier, most gap junction blockers, including carbenoxolone/glycyrrhetic acid-derived molecules also block hemichannels and are thus not hemichannel-specific (Takeuchi et al., 2011). Lanthanum ions (La^{3+}) block hemichannels and do not inhibit gap junctions (Anselmi et al., 2008) but this trivalent ion also blocks Ca^{2+} channels (Mlinar & Enyeart, 1993; Young et al., 2002). Gadolinium (Gd^{3+}) is another trivalent ion hemichannel blocker (Stout et al., 2002). These trivalent ions can be used for *in vitro* work on cells with low expression of Ca^{2+} channels but they are not appropriate tools to block hemichannels in a complex *in vivo* setting. Gap26/27 peptides are also useful, provided cell exposure is short enough (tens of minutes) to prevent effects on gap junctions. Peptide5 (VDCFLSRPTEKT (Figure 1) [see Table 1], not to be confused with peptide5 from the Warner et al. (1995a) study, overlaps with Gap27 (SRPTEKT) but lacks the last 4 C-terminal amino acids and has 5 extra amino acids N-terminally. This peptide was identified by testing various slightly overlapping mimetic sequences of the Cx43 EL2 for their potential to inhibit the swelling of rat pup spinal cord segments kept in organotypic culture; peptide5 more than halved swelling after 1 or 4 days in culture. Propidium iodide hemichannel dye uptake assays with a low extracellular Ca^{2+} concentration showed hemichannel block at 5 μM while calcein dye spread studies indicated gap junction block at 500 μM (O'Carroll et al., 2008). Interestingly, peptide5 also reduced Cx43 expression and had beneficial effects on glial fibrillary acidic protein (GFAP) expression in astrocytes and neuronal nuclei (NeuN) and SMI-32 (marker of nonphosphorylated neurofilaments) expression. The distinct concentration-dependence of hemichannel and gap junction block indicates there may be two different, yet unknown interaction sites for peptide5. It should be noted that, although peptide5 mimics a sequence on EL2 of Cx43, this does not mean that this peptide is necessarily selective for this particular Cx – further testing of its selectivity is therefore indicated.

Recent work has identified two new peptides, called L2 and Gap19, (Figure 1) which specifically block Cx43 hemichannels without inhibiting gap junctions. In contrast to Gap26/27 and peptide5, these two peptides mimic sequences located at the intracellular side of the Cx43 protein which is known to vary widely between different connexins. The L2 sequence is located on the cytoplasmic loop (CL) of Cx43 and the Gap19 sequence is a 9 amino acid stretch within the L2 domain (Wang et al., 2013) (Table 1)(L2 sequence: DGANVDMHLKQIEIKKFKYGIEEHGK, Gap19 sequence underlined). The L2 region is involved in CL interactions with the CT and the short Gap19 sequence is a crucial domain for this interaction. CT-CL interaction results in closure of gap junctions, according to a putative ball-and-chain model (reviewed in (Delmar et al., 2004)). Exogenous addition of L2-derived RXP Cx43-binding peptides prevents CT-CL interaction and thereby prevents

gap junction closure induced by acidification or octanol (Verma et al., 2009), i.e., L2 peptide acts to stabilize the open state of gap junctions. Surprisingly, the L2 peptide, in contrast to its effects on gap junctions, blocks Cx43-based hemichannels (Ponsaerts et al., 2010). Gap19 displays similar effects: inhibition of Cx43 hemichannels while not affecting gap junctions upon short exposure (30 min) but slightly stimulating them upon longer exposures (24-48 hrs) (Wang et al., 2013). In depth mechanistic investigations based on single-channel patch clamp experiments, surface plasmon resonance (SPR) and amino acid substitution experiments showed that CT-CL interactions are necessary for hemichannel opening (Abudara et al., 2014; D'Hondt et al., 2013; Iyyathurai et al., 2013; Ponsaerts et al., 2012; Ponsaerts et al., 2010; Ponsaerts et al., 2012; Wang et al., 2013). CT-CL interactions by themselves do not trigger hemichannel opening *per se* but bring the hemichannels into an available-to-open state that allows their opening by other stimuli of electrical or chemical nature (Saez & Leybaert, 2014; Wang et al., 2013). Moreover, Gap19 specifically acts on Cx43 hemichannels while not influencing Cx40 hemichannels or pannexin 1 channels (Wang et al., 2013). Thus, Gap19 and L2 peptides are interesting tools to investigate contributions of Cx43 hemichannels in brain and heart diseases, in particular ischemia and/or inflammatory conditions. L2 is not membrane permeant but Gap19 has some intrinsic plasma membrane permeability related to its high lysine content (4 out of 9 amino acids are lysines). Both Gap19 and L2 peptides can be linked to the TAT membrane translocation motif to improve/facilitate cell entry and access to the cytoplasmic Cx43 CT target. Determination of the half-maximal hemichannel inhibition concentration for the TAT-linked versions of these peptides gave values of ~10 μM for TAT-L2 and ~7 μM for TAT-Gap19, which are in good agreement to the K_d values for both peptides to interact with the CT determined from SPR experiments. Other novel interesting molecules, for example the linear peptide RRNYRRNY, the cyclic peptide CyRP-71 and the peptidomimetic ZP2519 target the Cx43 CT and act to prevent Cx43-based gap junction closure (De Vuyst et al., 2011; Verma et al., 2009; Verma et al., 2010). These substances are of potential translational value because they prevent gap junction closure and thereby preserve the physiological roles of gap junctions while they are expected (but still not proven) to inhibit hemichannel opening (by preventing CT-CL interaction).

Another approach for blocking hemichannels is the use of antibodies directed against the extracellular portions of the Cx proteins. Antibodies against the ELs have been generated for Cx26 (Clair et al., 2008) and Cx43 (Baklaushev et al., 2009; Riquelme et al., 2013; Hofer & Dermietzel, 1998; Siller-Jackson et al., 2008). Antibodies raised against the EL2 of Cx43 (Figure 1) have higher affinity than those against EL1, hence EL2 antibodies have been most frequently used. EL2 antibodies have been raised against the sequence T186 – K206 (polyclonal Ab, (Hofer & Dermietzel, 1998)), Y185 – K206 (polyclonal Ab, (Siller-Jackson et al., 2008)) or Q173-I208 (monoclonal Ab, (Baklaushev et al., 2009)) which include part of the Gap27 sequence (SRPTEKTI for Q173-I208). These antibodies block hemichannels as determined in assays of low extracellular Ca^{2+} -triggered Lucifer Yellow dye uptake in astrocytes (Hofer & Dermietzel, 1998) and fluid shear stress-induced prostaglandin E_2 release in osteocytes (Siller-Jackson et al., 2008). Because they are bulky, it is conceivable that hemichannel block is caused by pore obstruction. Additionally, EL-antibodies invariably also inhibit gap junctions: they interact with the ELs of free hemichannels and

thereby prevent the docking and formation of gap junctions (Riquelme et al., 2013). Like for Gap26/27 peptides, gap junction block occurs with some latency which likely depends on the turn-over rate of the Cx protein (antibody-bound hemichannels cannot form new gap junction channels resulting in slow disappearance of junctional coupling). Although EL-antibodies have significantly higher affinities than EL-peptides, they may suffer from the same specificity limitations because of the conserved nature of the EL sequences (for example: Cx32 EL2 has a homology score of ~70% with Cx43 EL2, (Hofer & Dermietzel, 1998)). EL2 antibodies do however not block pannexin channels (Siller-Jackson et al., 2008) that have strongly different sequences compared to Cx.

4. Ischemia/reperfusion injury and neuroprotection in the central nervous system

4.1. Irreversible brain injury

Numerous studies of the effect of gap junction blockers and knockout of specific Cx have been performed on models of brain ischemia and central nervous system trauma. Most of the gap junction blockers used have off-target effects (see 5. Pharmacological modulation of Cx43-formed channels) and also affect hemichannels, making mechanistic interpretation of the reported results difficult.

Early studies using halothane reported a reduction in infarct size in a mid-cerebral artery occlusion (MCAO)/reperfusion model in rats and cats (Saito et al., 1997; Warner et al., 1995). Intraperitoneal injection of octanol 30 min before MCAO (without reperfusion) in rats reduced infarct size measured 24 hrs later by 50% (Rawanduzy et al., 1997). A similar octanol treatment schedule gave a 30-50% reduction of cell death in the hippocampus following transient (10 min) bilateral clamping of the common carotid arteries in rats (Rami et al., 2001). In a model of transient (12.5 min) intrauterine global hypoxia applied to at term pregnant rats, carbenoxolone administration reduced pup mortality by 40% 24 hrs later and by 55% 21 days later. Caspase-3 activation in the brain was reduced by 57% 24hrs after hypoxia (de Pina-Benabou et al., 2005). Using a model of transient (18 min) bilateral global ischemia (4 vessel occlusion), carbenoxolone and 18 α -glycyrrhetic acid administered 30-40 min before ischemia directly into the hippocampus reduced cell death by 27% in the CA1 region 5 days after ischemia (Perez Velazquez et al., 2006). Interestingly, treatment was also effective when substances were applied 30-40 min after ischemia induction but not when applied at 90 min post-induction, indicating that the first 1.5 hrs of ischemia/reperfusion are critical for protection by carbenoxolone/18 α -glycyrrhetic acid treatment.

While the previous studies with Cx channel blockers reported improved outcomes, subsequent studies in knockout animals showed the opposite result. Permanent MCAO gave a 83% larger infarct size and 120% larger rate of apoptosis (TUNEL) in the penumbra zone (zone around the ischemic core) when applied to global heterozygous Cx43 knockout mice as compared to wildtype mice (Nakase et al., 2003; Siushansian et al., 2001). Interestingly, astrogliosis was reduced in heterozygous Cx43 knockout mice, which might result from diminished astrocytic intercellular Ca²⁺ wave activity (De Bock et al., 2014). Follow-up work by the same group in astrocyte-specific Cx43 knockout mice confirmed these results:

larger infarct size and rate of apoptosis in the penumbra (Nakase et al., 2004). An up-regulation of astrocytic Cx30 (the second most prevalent Cx in astrocytes) in heterozygous Cx43 knockout mice was reported (Nakase et al., 2003) but no data were included on the effect of Cx30/Cx43 double knockout animals on infarct size and astrogliosis. The impact of CT-truncation of Cx43 (Cx43 K258stop; Cx43^{CT/+}) was studied in the MCAO model. Infarct volume was twice as large in Cx43^{CT/+} and Cx43^{CT/-} as compared to wildtype mice (Kozoriz et al., 2010). Astrogliosis was reduced by 30% (Cx43^{CT/+}) to 50% (Cx43^{CT/-}) and microglial infiltration of the peri-infarct zone was almost twice as large in Cx43^{CT/+} and Cx43^{CT/-} compared to wildtype mice. Interpretation of these results is more difficult, as the CT-truncation used affects channel as well as non-channel functions of Cx43. The effect of Cx43^{CT/+} in astrocyte cultures demonstrated slightly reduced gap junctional coupling in Cx43^{CT/+} and a more pronounced reduction in coupling (halving) in Cx43^{CT/-}. A subconductance state of gap junctional gating appeared to be lost. Hemichannels were also tested and propidium iodide dye uptake triggered by a low extracellular Ca²⁺ concentration suggested a doubling of hemichannel function in Cx43^{CT/+} and halved in Cx43^{CT/-} relative to wildtype mice. Intercellular Ca²⁺ waves, which are propagated by both gap junction and hemichannel related mechanisms (Leybaert & Sanderson, 2012) were normal in Cx43^{CT/+} but reduced in Cx43^{CT/-}. Overall, the infarct size was increased to a comparable degree in both Cx43^{CT/+} and Cx43^{CT/-} while the effect on channel functions was rather diverse. Although fine-detailed interpretation of these results has been proposed in terms of channel functions of Cx43 (Kozoriz et al., 2010), overall it looks like the increased brain infarct size associated with CT-truncation has more to do with an influence on non-channel functions of Cx43.

Remarkably, no reports are available investigating the use of Gap26/27 as potential protective agents against ischemic injury in the brain, while these peptides display clear cardioprotective potential (Hawat et al., 2010; Hawat et al., 2012). In part, this is related to the fact that these peptides cannot pass the blood-brain barrier (BBB) although they might pass through once the BBB has lost its integrity (molecular weight of Gap26 is 1551 and for Gap27 1305). Importantly, Cx in BBB endothelial cells are an accessible and promising target for neuroprotection purposes in their own right. For example, intravenously administered Gap27 protected the BBB against bradykinin-induced leakage by inhibiting oscillations in the endothelial cytoplasmic Ca²⁺ concentration (De Bock et al., 2011). Ca²⁺ entry via Cx hemichannels (Cx43 but probably also Cx37) contributed to endothelial Ca²⁺ oscillations and inhibiting this Ca²⁺ entry pathway with Gap27 preserved BBB function (De Bock et al., 2011; De Bock et al., 2012; De Bock et al., 2013). Peptide5, which has some sequence overlap with Gap27 (see Table 1) and blocks hemichannels at 5 µM concentration has been demonstrated to be a potential neuroprotectant in various *in vivo* models of ischemia in brain as well as in spinal cord injury. In most cases, application was done directly to the neuronal tissue to circumvent problems of limited BBB passage of this peptide. In a model of transient 30 min ischemia by bilateral clamping of the carotid arteries in fetal sheep, peptide5 when infused intraventrically 90 min after ischemia for 1 hr or 25 hrs, improved oligodendrocyte survival and prevented brain weight loss associated with ischemia (Davidson et al., 2012). Neuronal cell loss was partly but not significantly reduced. Making use of the same model, the authors compared intraventricular peptide5 infusion

starting 1 hr before and applied during ischemia or starting 90 min after ischemia and infused for the next 25 hrs. Only the latter post-ischemia treatment protected significantly against neuronal cell loss in the cortex and oligodendrocyte loss in various white matter regions (Davidson et al., 2013). Neuronal cell loss in the most vulnerable hippocampal CA1 and CA3 regions was however not prevented by any of those treatments. Overall, this work demonstrates that peptide5 action is most prominent after ischemia, presumably by preventing hemichannel opening and thereby limiting injury spread in gray and white matter during reperfusion. Intraperitoneal administration of peptide5 at the start of reperfusion (100 μ M estimated systemic concentration) limited Evans blue dye leakage from blood vessels 4 hrs and 24 hrs later and prevented vascular endothelial cell death after 6 hrs of reperfusion (Danesh-Meyer et al., 2012). GFAP astrocyte immunoreactivity was (non-significantly) reduced and Cx43 expression was doubled in astrocytes and endothelial cells starting from 4 hrs.

4.2. Spinal cord injury

In vivo data are also available from spinal cord injury models. The pathophysiology of spinal cord injury involves the mechanotrauma proper, associated with direct cell injury, as well as ischemia related to vascular thrombosis or tissue swelling-induced vessel compression, leading to a secondary/delayed phase of cell death. In a rat spinal cord compression model, Cx43 downregulation by local application of antisense oligodeoxynucleotides (Cx43-asODN) immediately after injury improved the locomotion score from 24 hrs after injury up to 4 weeks later (Cronin et al., 2008). It also decreased cord tissue swelling and disruption, gave less astrocyte activation, less extravasation of bovine serum albumin and less infiltration of neutrophils. In another spinal cord transection model, the observations of less astrocyte activation and neutrophil recruitment upon Cx43-asODN treatment were confirmed, and it was further demonstrated that treatment decreased OX42⁺ microglial cell recruitment to the lesion site. Animals with astrocyte specific Cx43 knockout combined with global knockout of Cx30 (Cx30/Cx43 double knockout) displayed a consistently improved locomotor score starting from post-injury day 3 and lasting for at least 56 days (Huang et al., 2012). Electrophysiological recovery estimated from compound action potential amplitude was also better in the Cx30/Cx43 double knockout mice. The lesion volume was more than halved at 8 weeks post-injury in the double knockout mice and the myelin mass was 3.5 times larger in the double knockout compared to wild-type mice. These are striking observations as astrocyte-specific Cx43 knockout results in a larger infarct size in brain ischemia as discussed above ((Nakase et al., 2004) – though no data are available on Cx30/Cx43 double knockout mice for brain ischemia). Counts of reactive astrocytes (GFAP positive) and microglial cells were also decreased (Huang et al., 2012). Interestingly, post-injury ATP release measured in the tissue was decreased in the double knockout and the authors suggested a link between suppressed ATP release and protection against neuronal and oligodendrocyte cell death as well as decreased astrocyte and microglial activation. Hemichannel blockade with peptide5 had similar effects as decreasing Cx43 expression. In a spinal cord weight drop rat model, local application of peptide5 for 24 hrs (applied via an intrathecal catheter and an osmotic pump) improved locomotor recovery, prevented the Cx43 increase at 4 hrs and 8 hrs post-injury and restored its phosphorylation at S368 (O'Carroll et al., 2013). This treatment also decreased astrocyte activation (GFAP

expression) adjacent to the lesion site and cell counts of activated microglia up to several millimeters away from the lesion site in gray and white matter. Neuron counts were also increased after treatment up to several mm away from the lesion (almost double counts of SMI32⁺ cells). Tumor necrosis factor- α and interleukin 1- β expression at the injury site was diminished by peptide5 treatment, suggesting a suppressive effect on inflammation and microglial activation via inhibition of ATP release or other mechanisms.

4.3. Conditioning

Ischemic preconditioning has been best characterized in the heart (see 7. Ischemia/reperfusion injury and protection in the heart) but also exerts protective effects in the brain (Gidday, 2006). Similar to the heart, the signaling cascade involves mitochondrial ATP-dependent potassium channels and K⁺ fluxes (Wu et al., 2011). However, in contrast to the heart where preconditioning is linked to mitochondrial Cx43, brain preconditioning has been linked to the opening of plasma membrane hemichannels, with subsequent ATP release and formation of extracellular adenosine (Lin et al., 2008). A transient MCAO (45 min) followed by reperfusion for 24 hrs had a reduced infarct volume when occlusion was preceded by ischemic preconditioning in wildtype mice (infarct volume more than halved) while there was no protection in Cx30/Cx43 double knockout animals (global knockout for Cx30, astrocyte specific knockout for Cx43).

In summary, although results using hemichannel inhibitors and genetic deletion of the hemichannel gene provided conflicting results, selective Cx43 hemichannel inhibition might be a promising target in neuroprotection.

5. Myocardial ischemia/reperfusion injury and cardioprotection

5.1. Irreversible injury

Cx43 forms hemichannels, which are predominantly closed in healthy myocardium (Goodenough et al, 2004; Krysko et al., 2005). However, they can open in response to electrical and chemical triggers, most notably during ischemia and inflammatory conditions (Saez et al., 2005; Saez & Leybaert, 2014; Wang et al., 2012a; Wang et al., 2013a; Wang et al., 2013b). In cardiomyocytes, metabolic inhibition activated a non-selective current through Cx43 hemichannels (Kondo et al., 2000). Single channel analysis of this current demonstrated a reversal potential of 0 mV and a unitary conductance of ~200 pS, which are biophysical properties compatible with Cx43 hemichannel activity (Wang et al., 2012a; Wang et al., 2013b). The 0 mV reversal potential suggests non-selectivity in terms of ion permeation. As a result, prolonged opening of Cx43 hemichannels during ischemia may lead to loss of ionic gradients, excessive Ca²⁺ entry, cell swelling and cellular damage. Cx43 hemichannels are also a well-defined release pathway for several metabolites such as ATP (Kang et al., 2008) and glutamate (Ye et al., 2003) to the extracellular space affecting homeostasis of neighboring cells (Tsukimoto et al., 2005).

Cardiomyocyte dye uptake following 1 hr simulated ischemia was blocked by Gap26 (Shintani-Ishida et al., 2007), and cardiomyocyte swelling caused by simulated ischemia/reoxygenation was blocked by Gap19 (Wang et al., 2013b). As a consequence, cardiomyocyte survival after simulated ischemia/reoxygenation was increased by Gap19

(Wang et al., 2013b) or Gap26 (Hawat et al., 2010). Also in mice hearts in vivo, infarct size after ischemia/reperfusion was attenuated by blockade of Cx43 hemichannels using Gap19 (Wang et al., 2013b). Gap26/Gap27 reduced myocardial infarct size after ischemia/reperfusion in rats (Hawat et al., 2010;Hawat et al., 2012;Johansen et al., 2011). The effect of Gap26/27 on infarct size appears to be larger than the one obtained with Gap19, which might be due to the fact that Gap26/27 is less selective towards gap junctions and hemichannels and also inhibits channels composed of Cx other than Cx43 (see 5. Pharmacological modulation of Cx43-formed channels).

Replacement of Cx43 by Cx32 in mice reduced infarct size following ischemia/reperfusion (Rodriguez-Sinovas et al., 2010) and also partial (50%) depletion of myocardial Cx43 attenuated infarct size in one (Kanno et al., 2003) but not in other studies (Heinzel et al., 2005;Schwanke et al., 2002). Thus, sustained ischemia/reperfusion opening of Cx43-formed hemichannels combined with residual gap junctional communication contributes to irreversible myocardial injury (the latter phenomenon has been termed “spreading of injury”, for a review, see (Garcia-Dorado et al., 2004)). Interestingly, in cultured cardiomyocytes, the rate of apoptosis increased rather than decreased when gap junctional communication was reduced (Yasui et al., 2000).

Many pharmacological interventions which reduce infarct size after ischemia/reperfusion such as blockade of p38 MAPK (Surinkaew et al., 2013), high density lipoprotein/ sphingosine-1 phosphate (Morel et al., 2012), PKC epsilon activating peptide (Lancaster et al., 2011), FGF2/diazoxide (Srisakuldee et al., 2009) or estradiol (Lee et al., 2004) preserved Cx43 phosphorylation during ischemia/reperfusion, although causality between altered Cx43 phosphorylation and reduced infarct size was proven only in one study (Srisakuldee et al., 2009).

5.2. Conditioning

In cardiomyocytes, preconditioning by simulated ischemia/reoxygenation reduced Lucifer Yellow uptake prior to the sustained ischemic period but maintained Lucifer Yellow uptake following the prolonged lethal simulated ischemia (Sundset et al., 2007). Maintained Lucifer Yellow uptake followed by prolonged simulated ischemia was associated with preserved Cx43 phosphorylation. Cardiomyocytes isolated from heterozygous Cx43 knockout mice – in contrast to cardiomyocytes from wildtype mice - could not be conditioned by simulated ischemia/reoxygenation (Li et al., 2004).

Similar to the data in isolated cardiomyocytes, infarct size reduction by ischemic (Sanchez et al., 2013;Schwanke et al., 2002) or pharmacological (Heinzel et al., 2005) preconditioning was absent in Cx43 knockout mice or in mice in which Cx43 was replaced by Cx32 (Rodriguez-Sinovas et al., 2010). Also blockade of Cx43-formed channels using heptanol during the preconditioning cycles of ischemia/reperfusion blocked the infarct size reduction in mice hearts (Li et al., 2002). In many studies, ischemic or pharmacological preconditioning increased the extent of phosphorylated Cx43 during sustained ischemia (Hatanaka et al., 2004; Hund et al., 2007; Miura et al., 2010; Miura et al., 2007; Naitoh et al., 2006;Naitoh et al., 2009; Totzeck et al., 2008;Jain et al., 2003; Lee & Chou, 2003; Schulz et al., 2003) and subsequently reduced gap junction permeability (Lee&Chou, 2003;

Miura et al., 2010; Miura et al., 2007; Naitoh et al., 2006; Naitoh et al., 2009) and increased lateralization of Cx43 (Vetterlein et al., 2006). Preserved phosphorylation was caused by activated PKC epsilon (Jain et al., 2003; Lee and Chou, 2003; Miura et al., 2010; Naitoh et al., 2009), and knockout of PKC abolished both the reduction in infarct size and the preservation of Cx43 phosphorylation by ischemic preconditioning in mice hearts (Hund et al., 2007). Apart from PKC, MAPK also contributed to Cx43 phosphorylation maintenance during sustained ischemia by ischemic or pharmacological preconditioning (Naitoh et al., 2006; Schulz et al., 2003). In some studies, however, the rate of Cx43 during sustained ischemia remained unaffected by ischemic preconditioning (Muhlfeld et al., 2010) and rate of gap junction uncoupling was decreased (Jain et al., 2003).

Importantly, although Cx43 deficient mice cannot be preconditioned by ischemic preconditioning, the RISK and SAFE pathways, which have been often associated with cardioprotection, became activated in these mice to a similar extent as in wildtype and conferred a protective effect (Sanchez et al., 2013). Indeed, the lack of cardioprotection by preconditioning in Cx43 deficient mice appears to be related to mitochondrial Cx43 and the insufficient mitochondrial production of reactive oxygen species (Heinzel et al., 2005).

Similarly to ischemic preconditioning, ischemic or pharmacological postconditioning reduced infarct size and preserved myocardial Cx43 expression and phosphorylation during sustained ischemia in rat hearts (Wu et al., 2012; Zhao et al., 2013). In contrast, to ischemic preconditioning, however, infarct size was reduced by ischemic postconditioning in heterozygous Cx43 knockout mice (Heusch et al., 2006) (for review, see (Schulz et al., 2007)).

Remote ischemic preconditioning (i.e. the preconditioning ischemia/reperfusion occurs in an organ distant from the heart) in rats preserved Cx43 protein expression and phosphorylation potentially contributing to the protection of the rat heart in vivo (Brandenburger et al., 2014).

In summary, blockade of Cx43 prior to ischemia and/or reperfusion reduces irreversible injury following ischemia/reperfusion. However, data obtained from ischemic conditioning studies suggest that Cx43 is essential also for cardioprotection. Thus, timing of blockade of Cx43 appears to be of utmost importance in order to maximize the cardioprotective effect.

6. Mitochondrial Cx 43

Apart from being present at the sarcolemma, Cx43 has been identified in mitochondria of cardiomyocytes (Boengler et al., 2005), astrocytes (Kozoriz et al., 2010a), endothelial cells (Kiec-Wilk et al., 2012; Mohammad & Kowluru, 2011; Trudeau et al., 2012), stem cells (Lu et al., 2012), hepatocytes (Vinken et al., 2013) and pancreatic cancer cells (Sun et al., 2012).

In cardiomyocytes, Cx43 is mainly located in the inner mitochondrial membrane of subsarcolemmal mitochondria (Figure 2; Boengler et al., 2009; Rodriguez-Sinovas et al., 2006), although one study reported Cx43 being present also in the outer mitochondrial membrane (Goubaeva et al., 2007). Mitochondrial Cx43 expression in the heart decreased with age (Boengler et al., 2007). A decline in mitochondrial Cx43 content was also seen

after activation of the NMDA receptor 1 (NMDAR-1) in cardiomyocytes; NMDAR-1 activation increased translocation of matrix metalloproteinase 9 to mitochondria thereby inducing mitochondrial Cx43 degradation and mitophagy (Tyagi et al., 2010). [A similar effect was also seen in retinal endothelial cells (Mohammad & Kowluru, 2011; Trudeau et al., 2012)]. In contrast, ischemia/reperfusion (Boengler et al., 2005;Penna et al., 2009) or diazoxide (Yang et al., 2011) increased cardiac mitochondrial Cx43 content.

Mitochondrial Cx43 is involved in loss of cardiomyocyte division after birth; the signaling cascade involves reactive oxygen species formation and p38 MAPK activation (Matsuyama & Kawahara, 2011). Knockdown of mitochondrial Cx43 or inhibition of p38 MAPK restored the proliferation of neonatal rat cardiomyocytes (Matsuyama and Kawahara, 2009).

Mitochondrial connexin 43 impacts on respiratory function (Boengler et al., 2012;Ruiz-Meana et al., 2014), and the ischemia-associated reduction in mitochondrial respiration is associated with increased oxidized Cx43 cysteines. The latter effects are attenuated by ischemic preconditioning (Ruiz-Meana et al., 2014). Apart from its effect on respiration, mitochondrial Cx43 modulates the matrix potassium flux in heart (Boengler et al., 2013;Miro-Casas et al., 2009) and brain (Kozoriz et al., 2010a) mitochondria. S-nitrosation of mitochondrial Cx43 increases mitochondrial permeability, especially for potassium, and leads to increased reactive oxygen species formation. The increased amount of S-nitrosation mitochondrial Cx43 by ischemic preconditioning or nitrite administration may link nitric oxide and Cx43 in the signal transduction cascade of cardioprotective interventions (Soetkamp et al., 2014).

Regulation of the matrix calcium content also depends on mitochondrial Cx43; the calcium retention capacity of subsarcolemmal mitochondria was reduced by blocking Cx43-formed channels with Gap27 (Srisakuldee et al., 2014). In mitochondria derived from rat brain, carbenoxolone reduced the calcium retention capacity (Azarashvili et al., 2011). The modification of the calcium retention capacity is always accompanied by alterations in the phosphorylation status of mitochondrial Cx43 with the S262 epitope being of particular importance (Azarashvili et al., 2011;Srisakuldee et al., 2014).

Abolishing the mitochondrial import of Cx43 by blocking its interaction with heat shock protein 90 completely blocked the infarct size reduction by diazoxide in isolated rat hearts (Miro-Casas et al., 2009). In this study, sarcolemmal Cx43 remained unaffected.

These data indicate that the presence of mitochondrial Cx43 is of utmost importance for cardioprotective pathway(s) being functional.

7. Cx43 function in the presence of major cardiovascular risk factors

It has been well established that organoprotective cellular mechanisms are inhibited by the presence of major cardiovascular risk factors (see for reviews: Ferdinandy et al., 2007, 2014). Indeed, aging, hypertension, and metabolic diseases have been shown to interfere with Cx43 signalling.

7.1. Age and gender

In aged mice and rat hearts, total and mitochondrial Cx43 expression are decreased (Boengler et al., 2007; Fannin et al., 2014; Watanabe et al., 2004; Zaman et al., 2014). Also lateralization of Cx43 is enhanced in aged cardiomyocytes (Dhein and Hammerath, 2001). The increased heterogeneity of Cx43 distribution correlates with age-associated alterations in heart rhythm and increased atrial fibrillation in patients (Nattel et al. 2007; Fannin et al., 2014). There are differences regarding gender as Cx43 expression is higher in female compared to male hearts (Knezl et al., 2008; Tribulova et al., 2005). Starvation in mice reduced the expression of Cx43 at the sarcolemma of cardiomyocytes (McLachlan et al., 2009).

7.2. Hypertension and cardiac hypertrophy

Most studies related to hypertension are done in spontaneously hypertensive rats. The results obtained differ in terms of Cx43 expression with Cx43 being up-regulated and phosphorylation of Cx43 being increased (Bacova et al., 2010; Bacova et al., 2012; Benova et al., 2013; Fialova et al., 2008; Mitasikova et al., 2008; Radosinska et al., 2011; Radosinska et al., 2013; Zhao et al., 2008) in some studies and Cx43 expression being decreased (Bacharova et al., 2008; Zhang et al., 2014) in others. Part of the observed differences might relate to the degree of left ventricular hypertrophy associated with hypertension, since in human hearts mild hypertrophy increased while extensive hypertrophy reduced left ventricular Cx43 expression (Kostin et al., 2004). Mild left ventricular hypertrophy due to pressure or volume overload was associated with changes in Cx43 sarcolemmal distribution in human hearts (Vetter et al., 2010). Similarly in mice with aortic banding, total Cx43 expression remained unaltered but Cx43 became redistributed (Boulaksil et al., 2010) or total Cx43 expression decreased (Qu et al., 2009; Yasuno et al., 2013) and Cx43 was dephosphorylated (Yasuno et al., 2013). Indeed, many interventions which reduce hypertension and/or hypertrophy (angiotensin II (AT) receptor 1-blockade (Zhao et al., 2008) or knockout (Yasuno et al., 2013), aldosterone antagonists (Yasuno et al., 2013), renin inhibition (Zhang et al., 2014) but also compounds such as melatonin (Benova et al., 2013), atorvastatin (Bacova et al., 2010; Chen et al., 2007), omega-3 free fatty acids (Bacova et al., 2012) or red palm oil (Mitasikova et al., 2008)) attenuate the observed changes in Cx43 expression and/or phosphorylation. Interestingly, blockade of AT- receptor 1 attenuated microRNA-1 expression (Curcio et al., 2013) which subsequently affected Cx43 expression: an increase in microRNA-1 secondary to p38MAPK activation in cardiomyocytes (Zhang et al., 2010b) or overexpression of microRNA-1 in mice depressed Cx43 expression (Zhang et al., 2010b) but a decrease in microRNA-1 increased Cx43 expression in female hearts (Stauffer et al., 2011).

7.3. Diabetes and hypercholesterolemia

Cx43 expression under diabetic conditions also shows variability from being increased (Anna et al., 2014; Howarth et al., 2008; Joshi et al., 2014), unaltered (Nygren et al., 2007) or even decreased (Lin et al., 2006a; Lin et al., 2006b; Sheu et al., 2007). In contrast, data on Cx43 phosphorylation and distribution are more homogenous: almost all studies show an increase in Cx43 phosphorylation (Anna et al., 2014; Howarth et al., 2008; Lin et al., 2006a;

Lin et al., 2006b) [at PKC phosphorylation sites (Lin et al., 2006a; Lin et al., 2006b)] and a redistribution/lateralization of Cx43 in cardiomyocytes (Anna et al., 2014; Howarth et al., 2008; Joshi et al., 2014; Lin et al., 2006a; Lin et al., 2006b; Nygren et al., 2007), the latter being associated with an increase also in Cx43 nitrosation (Joshi et al., 2014).

In rabbits with hypercholesterolemia, JNK2 was activated which subsequently led to a reduction in Cx43 expression and redistribution of Cx43 away from gap junctions (Lin et al., 2005). Similarly in rats fed a high fat-diet, myocardial Cx43 expression was reduced and Cx43 was redistributed away from gap junctions and mitochondria in cardiomyocytes (Gorbe et al., 2011).

7.4. Post-myocardial infarction

There is a substantial amount of literature demonstrating a reduction and disorganization of myocardial Cx43 in the infarcted area as well as in the border zone. The decrease in myocardial Cx43 expression post-myocardial infarction in mice (Jackson et al., 2008; Lindsey et al., 2006) was related to increased NO availability since Cx43 expression was preserved in nitric oxide synthase 2 knockout mice despite a similar extent of infarction (Jackson et al., 2008). In cardiomyocytes, a nitric oxide donor dose-dependently decreased Cx43 expression over time (Jackson et al., 2008). Part of the degradation of Cx43 appears to be through matrix metalloproteinase 7 since the reduction in Cx43 expression post-myocardial infarction was not detected in matrix metalloproteinase 7 knockout mice (Lindsey et al., 2006). Also an increase in micro RNA-1 in the infarcted heart (Yang et al., 2007) might contribute to the decline in myocardial Cx43 expression (see above). Interestingly, the amount of fibrosis post-myocardial infarction is reduced in heterozygous Cx43 knockout mice through decreases in phosphoSMAD despite increased TGF β concentrations (Zhang et al., 2010a), again pointing to non-channel effects of Cx43.

7.5. Heart Failure

In failing animal hearts, total Cx43 expression (Ai & Pogwizd, 2005; Danielson et al., 2013; Givvimani et al., 2014; Wang et al., 2012b) and Cx43 expression at gap junctions is reduced (Hesketh et al., 2010; Petrich et al., 2004; Wang et al., 2012b; Zhong et al., 2007) independent of the cause of heart failure. Similarly, in tissue samples from failing human hearts (dilated or ischemic cardiomyopathy), Cx43 expression is reduced and Cx43 is redistributed away from the gap junctions (Dupont et al., 2001; Kostin et al., 2003). In some heart failure studies, Cx43 became dephosphorylated and dye transfer was reduced (Ai et al., 2011; Ai & Pogwizd, 2005) while in one study the phosphorylation of the S255 residue of Cx43 increased (Sato et al., 2008). Overexpression of Cx43 in failing hearts through adenovirus application restored cardiomyocyte coupling (Ai et al., 2010). The decrease in Cx43 expression is related to increased mitophagy and matrix metalloproteinase activation and can be blocked by inhibiting mitochondrial division (Givvimani et al., 2014), but also an increase in micro RNA (19a/b) contributes to loss of Cx43 (Danielson et al., 2013). In viral myocarditis, increases in micro RNA-1 repressed Cx43 expression in mice (Xu et al., 2012).

In conclusion, cardiovascular risk factors often are associated with a Cx43 deficient state especially in mitochondria thereby interfering with cardioprotection.

10. Conclusion and perspectives

Cx43 has multiple cellular locations and functions and changes in its expression and/or its channel and non-channel activities contribute to several cardiovascular pathologies (brain and heart irreversible injury, arrhythmias). More research is needed to understand the pathophysiological alterations in Cx43 and to develop potential selective pharmacological approaches to reverse these effects. Development of mimetic peptides (opening/closing Cx43-formed channels) which may differentiate between the different channel subtypes at the sarcolemma (hemichannels, gap junctions) or mitochondria or interfere with the non-channel function of Cx43 might be a promising approach to help treating cardiovascular pathologies in the future.

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9. List of abbreviations

2-APB	2-aminoethoxydiphenyl borate
asODN	antisense oligodeoxynucleotides
ATP	adenosine triphosphate
BBB	blood-brain barrier
Ca²⁺	calcium
C-Src	tyrosine kinase cellular und sarcoma
CK	casein kinase
CL	cytoplasmic loop
CREB	cyclic adenosine monophosphate responsive element binding protein
CK1	casein kinase 1
CT	carboxy-terminal tail
Cx	connexin
Cx43	connexin 43
DIDS	disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate

EL	extracellular loop
Gd³⁺	Gadolinium
GFAB	Glial fibrillary acidic protein
GSK3β	glycogen synthase kinase 3 β
Hr(s)	hour(s)
IP₃	inositol triphosphate
JNK	c-Jun N-terminal kinase
K⁺	potassium
La³⁺	Lanthanum ions
MAPK	mitogen activated protein kinase
MCAO	mid cerebral artery occlusion
Min	minutes
MSX	Msh homeobox
Na⁺	sodium
NeuN	<i>neuronal</i> nuclei
NMDA	<i>N</i> -Methyl-d-aspartate
NPPB	5-Nitro-2-(3-phenyl-propylamino)benzoic acid
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
S	serine
SPR	surface plasmon resonance
TBX18	family member of T-box transcription factors
ZO-1	zona occludens proteins 1

14. List of References

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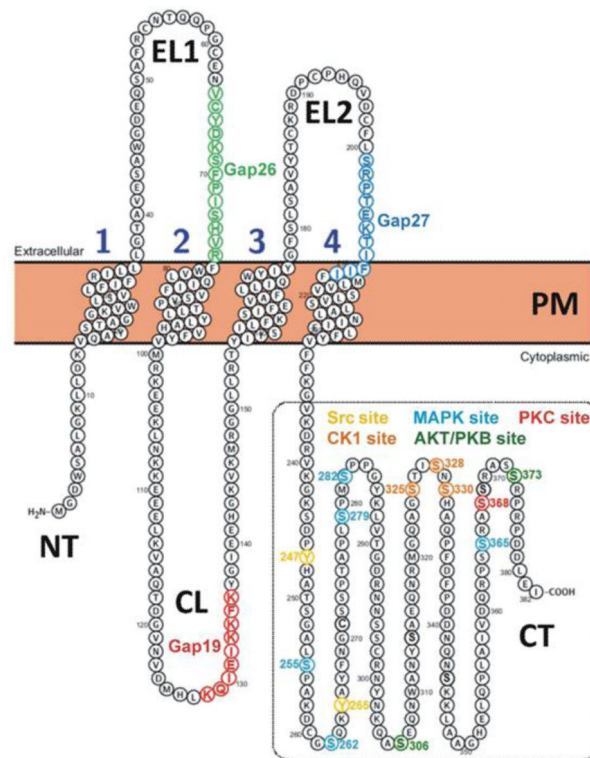


Figure 1. Schematic drawing of the arrangement of Cx43 in the plasma membrane (PM)
 Cx43 has a tetraspan topology with two extracellular loops (EL1 and EL2), one cytoplasmic loop (CL) and intracellular located N- and C-terminal tails (NT, CT). Prototypic Cx43 mimetic peptide sequences are indicated and localized on EL1 (Gap26), EL2 (Gap27) and CL (Gap19). The CT contains a large range of phosphorylation sites that are the target of the various kinases indicated. Illustration generated with the Protter tool (Omasits et al. 2014).

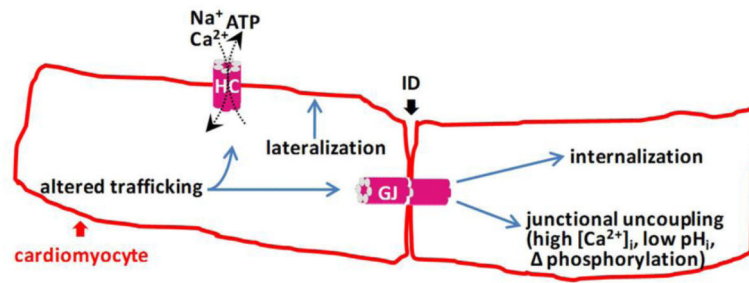


Figure 2. Connexin channels in cardiac ischemia

Connexins form gap junctions (GJ) that connect cardiomyocytes with each other at the site of cell-cell junction located intercalated disks (ID). They also form free unapposed hemichannels (HC) in the plasma membrane not incorporated in GJs. Ischemic conditions lead to junctional uncoupling and GJ closure as a result of $[Ca^{2+}]_i$ elevation (Dekker et al., 1996; Peracchia, 2004; Xu et al., 2012), acidosis (Ek-Vitorin et al., 1996; Ek et al., 1994), altered phosphorylation status (Ek-Vitorin et al., 2006; Pahuja et al., 2007) and other ischemia-related factors (Sanchez et al., 2011). In addition to this, connexins are remodeled as a result of processes that involve lateralization of connexin protein (Chkourko, et al., 2012; Kieken et al., 2009), altered trafficking (Remo et al., 2011; Smyth et al., 2010) and internalization (Duffy et al., 2004; Smyth et al., 2014; Sorgen et al., 2004). Most evidence comes from Cx43 which is a major connexin in ventricular cardiomyocytes that is also present in atria (in addition to Cx40). Cx43 hemichannels are normally closed but open in response to ischemia mimicking conditions (Kondo et al., 2000; Contreras et al., 2002), lowered redox status (Retamal et al., 2007; Saez et al., 2010), lowering of extracellular $[Ca^{2+}]$ (Li et al., 1996; Torres et al., 2012), moderate elevation (500 nM) of $[Ca^{2+}]_i$ (De Vuyst et al., 2009; Ponsaerts et al., 2010; Wang et al., 2012a) and mechanical stress (Batra et al., 2014). They open with alkalinization, close with acidosis (Schalper et al., 2010) and close with above 500 nM $[Ca^{2+}]_i$ elevation (Wang et al., 2012a). It is currently not clear how connexin remodeling impacts hemichannel function. GJ closure acts in a protective manner by limiting cell death spread to neighboring cardiomyocytes but may also lead to increased propensity for postischemic arrhythmogenesis because of heterogeneities in conduction velocity and consequent conduction delays. Hemichannel opening may lead to excessive entry of Na^+ and Ca^{2+} and the loss of essential metabolites (ATP and others) from the cells (Saez et al., 2010).

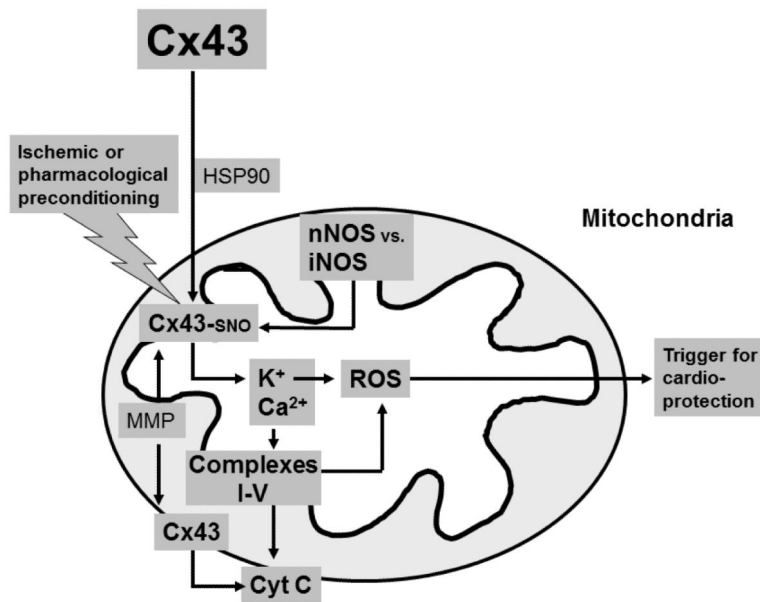


Figure 3. Cx43 in mitochondria (Cx43)

Cx43 is imported into the inner mitochondrial membrane of mitochondria in a heat shock protein (HSP) 90– dependent pathway. Its import into subsarcolemmal mitochondria in cardiomyocytes is increased by preconditioning interventions. Cx43 is S-nitrosylated and influences mitochondrial matrix potassium (K^+) and calcium (Ca^{2+}) influx and reactive oxygen species (ROS) formation. Mitochondrial Cx43 is important for cardioprotection through modification of ROS formation. Apart from its influence on ion fluxes, Cx43 affects mitochondrial respiratory complex function. Some studies indicate that Cx43 might also be localized at the outer mitochondrial membrane where it is important for cytochrome C (Cyt C) release. Finally, matrix metalloproteinases (MMP) affect the stability of mitochondrial Cx43. For details see text.

Table 1

Connexin mimetic peptides

Peptide	Cx43 sequence (human)
Gap26	VCYDKSFPISHVR
Gap27	SRPTEKTIFII
Peptide5	VDCFLSRPTEKT
L2	DGVNVDMHLKQIEIKKFKYGIEEHGK
Gap19	KQIEIKKFK

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Table 2

Importance of connexin 43 in ischemia/reperfusion injury and protection from it.

	Brain		Spinal cord	Heart	
	Ischemia	Conditioning	injury	Ischemia	Conditioning
Cx43 ^{+/-}	↑			↓ or =	Protection lost
Cx43 ^{-/-}	↑	Protection lost	Function improved (Cx43/30 double KO)	↓ (Cx32 KI)	Protection lost (Cx32 KI)
Cx43K258	↑				
Cx43 antisense			Function improved		
Pharmacological Blockade	↓				Protection lost
Peptide5	↓		↓ Inflammation decreased		
Gap19				↓	
Gap26/27				↓	

Legend to Table: Arrows illustrate effect on irreversible injury (↑ = increased; ↓ = decreased) KO: knockout; KI: knockin. For details see text.

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