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The ClC-3 chloride channels in cardiovascular disease

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Abstract

ClC-3 is a member of the ClC voltage-gated chloride (Cl^-) channel superfamily. Recent studies have demonstrated the abundant expression and pleiotropy of ClC-3 in cardiac atrial and ventricular myocytes, vascular smooth muscle cells, and endothelial cells. ClC-3 Cl^- channels can be activated by increase in cell volume, direct stretch of β1-integrin through focal adhesion kinase and many active molecules or growth factors including angiotensin II and endothelin-1-mediated signaling pathways, Ca^{2+}/calmodulin-dependent protein kinase II and reactive oxygen species. ClC-3 may function as a key component of the volume-regulated Cl^- channels, a superoxide anion transport and/or NADPH oxidase interaction partner, and a regulator of many other transporters. ClC-3 has been implicated in the regulation of electrical activity, cell volume, proliferation, differentiation, migration, apoptosis and intracellular pH. This review will highlight the major findings and recent advances in the study of ClC-3 Cl^- channels in the cardiovascular system and discuss their important roles in cardiac and vascular remodeling during hypertension, myocardial hypertrophy, ischemia/reperfusion, and heart failure.

Keywords

ClC; chloride channels; heart disease; apoptosis; oxidative stress; remodeling; hypertension

Introduction

ClC-3 is a member of the ClC voltage-gated chloride (Cl^-) channel gene superfamily[1]. In 1994, ClC-3 cDNA was first cloned from rat kidney by Kawasaki *et al* using a polymerase chain reaction (PCR) cloning strategy[2]. ClC-3 is also abundantly expressed in brain[3], lung, kidney[4], heart[5, 6], and vasculature[7] of many species, including human[4, 6, 8]. Expression of the cloned rat ClC-3 yielded an outwardly-rectifying Cl^- current in *Xenopus* oocytes[2] and in somatic cell lines[3], which was completely inhibited by activation of protein kinase C (PKC)[2] or increased intracellular Ca^{2+} concentration[3]. The Cl^- currents produced by expression of cardiac ClC-3 in mammalian cells are also outwardly-rectifying and inhibited by PKC and share many biophysical and pharmacological characteristics with the volume-regulated Cl^- currents ($I_{Cl,\,vol}$) in cardiac myocytes[5, 9, 10–13], vascular smooth muscle cells[7], and many other cell types[14–16]. ClC-3 Cl^- channels can be activated also by direct stretch of β1-integrin through focal adhesion kinase and many active molecules or growth factors including angiotensin II (Ang II) and endothelin-1 mediated signaling pathways[17–20], Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[21] and reactive oxygen species[22, 23]. In the past 15 years, accumulated experimental data has shown that ClC-3 proteins are expressed in sarcolemmal membranes and intracellular organelles of cardiac myocytes, vascular smooth muscle cells, and endothelial cells[24–27]. Numerous

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studies have demonstrated the pleiotropy of ClC-3 in many cellular functions, including 1) as a key component of the volume-regulated Cl^- channels (VRCCs) to strengthen the regulatory volume decrease (RVD) and protect cardiac myocytes from excessive increase in cell volume during hypoxia, ischemia, or hypertrophy; 2) as a regulator of the redox signaling pathway through interaction with NADPH oxidase (Nox) and/or as a superoxide anion ($O_2^{\cdot -}$) transporter to improve myocyte viability against oxidative damage; 3) as an anti-apoptotic mechanism through regulation of cell volume and intracellular pH; and 4) as a regulator of other transport functions involved in the etiology of myocardial damage, heart failure, and hypertension (Figure 1).

This review will highlight the major findings and recent advances in the study of ClC-3 Cl^- channels in the cardiovascular system and discuss their important roles in cardiac and vascular remodeling during hypertension, myocardial ischemia/reperfusion, hypertrophy, and heart failure.

ClC-3 and VRCCs

Under osmotic, metabolic, and/or oxidative stress mammalian cells are able to precisely maintain their size through the regulated loss or gain of intracellular ions or other osmolytes to avoid excessive alterations of cell volume that may jeopardize structural integrity and a variety of cellular functions[28–31]. Even under physiological conditions, volume constancy of any mammalian cell is challenged by the transport of osmotically active substances across the cell membrane and alterations in cellular osmolarity by metabolism[28]. Thus, the continued operation of cell volume regulatory mechanisms, such as activation of VRCCs, is required for cell volume homeostasis in many mammalian cells, including cardiac myocytes and vascular smooth muscle cells (VSMCs)[12, 24, 32, 33]. Acute increase in cell volume (or cell swelling) initiates the regulatory volume decrease (RVD) process to bring the cells back to their initial volume, which is achieved by the opening of VRCCs and other channels and transporters mediating Cl^-, K^+, and taurine efflux that in turn drives water exit[28].

Although the exact identification of the protein(s) responsible for VRCCs has proven to be elusive, ClC-3 has been proposed to be the molecular correlate of the native VRCCs in cardiac myocytes[5] and VSMCs[7]. But the role of ClC-3 as a constituent of native VRCCs became an issue of debate owing to inconsistent and conflicting data collected from some laboratories[34–36]. Specially, the presence of the native VRCCs in two different cell types from the global ClC-3 knockout (*Clcn3*$^{-/-}$) mice[35] casts considerable doubt on the role of ClC-3 as a molecular component of VRCCs. However, later additional experiments using *Clcn3*$^{-/-}$ mice revealed that the properties of native VRCCs in the *Clcn3*$^{-/-}$ heart were significantly altered and the expression of a variety of membrane proteins other than ClC-3 was also markedly changed, raising fundamental questions about the usefulness of the *Clcn3*$^{-/-}$ mouse model to assess ClC-3 function[37]. A series of recent independent studies from many laboratories further strongly corroborated the hypothesis that ClC-3 encoded a key component of the native VRCCs in a variety cell types ranging from normal cardiac myocytes to cancer cells[16, 20, 37–46]. Knockdown of ClC-3 by siRNA[41, 42, 47], shRNA[48, 49], and antisense[20, 39, 43, 45] and intracellular dialysis of anti-ClC-3 antibody (Ab)[16, 37, 38, 44–46] all consistently eliminated VRCC currents in many types of cells. A recent study using the inducible heart-specific ClC-3 knockout mouse found that a time-dependent inactivation of *ClC-3* gene expression was correlated with an elimination of the endogenous VRCCs (Figure 2) and significantly compromised cardiac function (Figure 2)[50]. Therefore, ClC-3 remains a strong and viable candidate for VRCCs in the heart and may contribute to normal cardiac function.

VRCCs and ClC-3 in cardioprotection induced by ischemic preconditioning (IPC)

Ischemic preconditioning (IPC) is a phenomenon in which brief episodes of ischemia dramatically reduce myocardial infarction caused by a subsequent sustained ischemia[51]. IPC has an early phase (lasting 1–2 h) and a late phase or "second window" (lasting 24–72 h) of protection[52]. It has been reported that the block of $I_{Cl,\ swell}$ in rabbit cardiac myocytes inhibits IPC by brief ischemia, hypo-osmotic stress[53, 54] and adenosine receptor agonists[55]. These studies were solely based on the use of several Cl^- channel blockers, such as anthracene-9-carboxylic acid (9-AC) and 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS). These pharmacological tools lack specificity to a particular Cl^- channel in the heart and may also act on other ion channels or transporters[56, 57]. Therefore the causal role of $I_{Cl,\ swell}$ in IPC has been very difficult to be confirmed[58]. To specifically test whether the VRCCs are indeed involved in IPC, we have recently established *in vitro* and *in vivo* models of early IPC and late IPC in *ClCn3*$^{-/-}$ mice. Our preliminary results indicate that targeted inactivation of *ClC-3* gene prevented protective effects of late IPC but not of early IPC, suggesting that ClC-3/VRCCs may contribute differently to early and late IPC[59,60]. The underlying mechanisms for these differential effects are currently unknown. Recent reports, however, suggest that VRCCs and ClC-3 may play an important role in apoptosis[61] and inflammation[62]. Cl^- channel blockers DIDS and NPPB were as potent as a broad-spectrum caspase inhibitor in preventing apoptosis and elevation of caspase-3 activity and improved cardiac contractile function after ischemia and *in vivo* reperfusion[63]. Transgenic mice overexpressing Bcl-2 in the heart had significantly smaller infarct size and reduced apoptosis of myocytes after ischemia and reperfusion[64]. It has been shown that Bcl-2 induces up-regulation of $I_{Cl,\ vol}$ by enhancing ClC-3 expression in human prostate cancer epithelial cells[65]. Cell shrinkage is an integral part of apoptosis, suggesting that $I_{Cl,vol}$ and ClC-3 might be intimately linked to apoptotic events through regulation of cell volume homeostasis[61, 65, 66].

VRCCs and ClC-3 in myocardial hypertrophy and heart failure

Structural remodeling of myocardial hypertrophy and dilated cardiomyopathy involves oxidative stress and hypertrophic cell volume increase or dilated myocyte membrane stretch, which alters cell volume homeostasis and many cellular functions including cell proliferation, differentiation, and apoptosis. $I_{Cl,\ swell}$ is persistently activated in ventricular myocytes from a canine pacing-induced dilated cardiomyopathy model[67]. Using the perforated patch-clamp technique, Clemo *et al* found that, even in isotonic solutions, a large 9-AC-sensitive, outwardly rectifying Cl^- current was recorded in failing cardiac myocytes but not in normal cardiac myocytes. Graded hypotonic cell swelling (60%–90% hypotonic) failed to activate additional current while graded hypertonic cell shrinkage caused an inhibition of the "basal" Cl^- current in failing myocytes. Moreover, the maximum current density of the $I_{Cl,\ swell}$ in failing myocytes was about 40% greater than that in osmotically swollen normal myocytes. Constitutive activation of $I_{Cl,\ swell}$ is also observed in several other animal models of heart failure, such as a rabbit aortic regurgitation model of dilated cardiomyopathy[68], a dog model of heart failure caused by myocardial infarction[69], and a mouse model of myocardial hypertrophy by aorta binding[70]. In human atrial myocytes obtained from patients with right atrial enlargement and/or elevated left ventricular end-diastolic pressure, a tamoxifen sensitive $I_{Cl,\ swell}$ was also found to be persistently activated[67]. Therefore, it is possible that persistent activation of $I_{Cl,\ swell}$ is a common response of cardiac myocytes to hypertrophy or heart failure-induced remodeling.

The mechanism for persistent activation of $I_{Cl,\ swell}$ in hypertrophied or failing cardiac myocytes is still not clear. Perhaps the increase in cell volume caused by hypertrophy and

the stretch of cell membrane caused by dilation are both involved in the activation of $I_{Cl,\ swell}$. Alternatively, the persistent activation of $I_{Cl,\ swell}$ may be caused by signaling cascades activated during hypertrophy independent of changes in cell length and volume, or both. $I_{Cl,\ swell}$ could be activated by direct stretch of β1-integrin through focal adhesion kinase (FAK) and/or Src[49]. Mechanical stretch of myocytes also releases Ang II, which binds to AT1 receptors (AT1R) and stimulates FAK and Src in an autocrine-paracrine loop. A recent study by Browe and Baumgarten suggests that the stretch of β1-integrin in cardiac myocytes activates $I_{Cl,\ swell}$ by activating AT1R and NADPH oxidase and, thereby, producing reactive oxygen species (ROS). In addition, a potent NADPH oxidase inhibitor, diphenyleneiodonium (DPI), and a structurally unrelated NADPH oxidase inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), rapidly and completely blocked both background and stretch-activated Cl^- currents in cardiac myocytes[19]. Therefore, NADPH oxidase may be intimately coupled to the channel responsible for $I_{Cl,\ vol}$, providing a second regulatory pathway for this channel through membrane stretch or oxidative stress[19]. This finding is very important for further understanding of the mechanism for hypertrophy activation of $I_{Cl,\ swell}$ and ClC-3 channels and their relationship with hypertrophy and heart failure as it is very well known that Ang II plays a crucial role in myocardial hypertrophy and heart failure[71]. Interestingly, Miller and colleagues recently found that Cl^- channel inhibitors and knockout of ClC-3 abolished cytokine-induced generation of ROS in endosomes and ROS-dependent NF-κB activation in vascular smooth muscle cells[37], suggesting a potential close interaction between NADPH oxidase and ClC-3 (Figure 1). In human corneal keratocytes and human fetal lung fibroblasts ClC-3 knockdown by a short hairpin RNA (shRNA) significantly decreased VRCC and lysophosphatidic acid (LPA)-activated Cl^- current ($I_{Cl,LPA}$) in the presence of transforming growth factor-β1 (TGF-β1) compared with controls, whereas ClC-3 overexpression resulted in increased $I_{Cl,\ LPA}$ in the absence of TGF-β1[72]. ClC-3 knockdown also resulted in a reduction of α-smooth muscle actin (α-SMA) protein levels in the presence of TGF-β1, whereas ClC-3 overexpression increased α-SMA protein expression in the absence of TGF-β1. In addition, keratocytes transfected with ClC-3 shRNA had a significantly blunted regulatory volume decrease response following hyposmotic stimulation compared with controls. These data not only confirm that ClC-3 is important in VRCC function and cell volume regulation, but also provides new insight into the mechanism for the ClC-3-mediated fibroblast-to-myofibroblast transition[15].

The functional and clinical significance of VRCCs in the hypertrophied and dilated heart is currently unknown. Using a mouse aortic binding model of myocardial hypertrophy, we have found that globally targeted disruption of *ClC-3* gene (*ClCn3*$^{-/-}$) accelerated the development of myocardial hypertrophy and the discompensatory process, suggesting that activation of $I_{Cl,\ vol}$ might be important in the adaptive remodeling of the heart during pressure overload[73]. Interestingly, heart failure was found to be accompanied by a reduced $I_{Cl,\ vol}$ density in rabbit cardiac myocytes[43]. Our recent studies on the conditional heart-specific ClC-3 knockout (*hsClcn3*$^{-/-}$) mice (Figure 3) support the crucial functional role of ClC-3 channels in the adaptive remodeling of the heart against pressure overload[72]. As shown in Figure 3, echocardiography revealed marked signs of myocardial hypertrophy (a significant increase in left ventricular mass LVM) and heart failure (a significant increase in LVIDs and reduction in IVSs, LVEF, and %FS) in the *hsClcn3*$^{-/-}$ mice compared to their age-matched wild-type control mice (Figure 3B). In addition, both left and right atria were significantly enlarged (Figure 3C). These data strongly suggest that ClC-3 may play an important role in maintaining normal structure and function of the mammalian heart.

VRCCs and ClC-3 in electrophysiology and electrical remodeling

Activation of VRCCs is expected to produce depolarization of the resting membrane potential and significant shortening of action potential duration (APD) because of its strong outwardly rectifying property[5, 11, 24, 74, 75]. The Cl^- current through the VRCCs under basal or isotonic conditions is small[10, 11, 76] but can be further activated by stretching of the cell membrane by inflation[77] or direct mechanical stretch of membrane β_1-integrin[78] and/or cell swelling induced by exposure to hypoosmotic solutions[5, 9–13]. The consequences of activation of $I_{Cl,\ vol}$ are very complex. It may be detrimental, beneficial, or both simultaneous in different parts of the heart, depending on environmental influences.

Because cardiac myocytes swell during hypoxia and ischemia, and the washout of hyperosmotic extracellular fluid after reperfusion induces further cell swelling, activation of VRCCs may contribute to APD shortening and arrhythmias induced by hypoxia, ischemia and reperfusion[79]. Shortening of APD and, therefore, the effective refractory period (ERP) reduces the length of the conducting pathway needed to sustain reentry (wavelength). In principle, this favors the development of atrial fibrillation (AF) or ventricular fibrillation (VF), depending on the presence of multiple reentrant circuits or rotating spiral waves. Activation of $I_{Cl,\ vol}$ may slow or enhance the conduction of early extrasystoles, depending on the timing. In guinea-pig heart, hypo-osmotic solution shortened APD and increased APD gradients between right and left ventricles. In burst stimulation-induced VF, exposure to hypo-osmotic solution increased VF frequencies, transforming complex fast Fourier transformation spectra to a single dominant high frequency on the left but not the right ventricle[19]. Perfusion with the VRCC blocker indanyloxyacetic acid-94 reversed organized VF to complex VF with lower frequencies, indicating that VRCC underlies the changes in VF dynamics. Consistent with this interpretation, ClC-3 channel protein expression is 27% greater on left than right ventricles, and computer simulations showed that insertion of $I_{Cl,\ vol}$ transformed complex VF to a stable spiral. Therefore, activation of $I_{Cl,\ vol}$ has a major impact on VF dynamics by transforming random multiple wavelets to a highly organized VF with a single dominant frequency.

In the case of myocardial hypertrophy and heart failure, ionic remodeling is one of the major features of pathophysiological changes[80]. Under these conditions, $I_{Cl,\ vol}$ is constitutively active[69]. The persistent activation of $I_{Cl,vol}$ may limit the APD prolongation and make it more difficult to elicit early after depolarization (EAD). Indeed, in myocytes from failing hearts, blocking $I_{Cl,\ vol}$ by tamoxifen significantly prolonged APD and decreased the depolarizing current required to elicit EAD by about 50%. And hyperosmotic cell shrinkage, which also inhibits $I_{Cl,\ vol}$, was almost equivalent to the effect of tamoxifen on APD and EAD in these myocytes[79]. It has been shown that mechanical stretching or dilation of the atrial myocardium is able to cause arrhythmias. Since $I_{Cl,\ vol}$ was also found in sinoatrial (S-A) nodal cells, VRCCs may serve as a mediator of mechanotransduction and play a significant role in the pacemaker function if they act as the stretch-activated channels in these cells[79, 81]. Baumgarten's laboratory has recently demonstrated that $I_{Cl,\ vol}$ in ventricular myocytes can be directly activated by mechanical stretch through selectively stretching β_1-integrins with mAb-coated magnetic beads[19, 79]. Although it has been suggested that stretch and swelling activate the same anion channel in some non-cardiac cells, further study is needed to determine whether this is true in cardiac myocytes and VSMCs.

VRCC and ClC-3 in vasculature and hypertensive vascular remodeling

It has been demonstrated that VRCCs and ClC-3 are expressed in aortic and pulmonary VSMCs of human and several other speicies[20, 82, 83] and have been implicated in a number

of vital cellular functions including vascular myogenic tone, cell volume regulation, cell proliferation and apoptosis[7, 26, 61, 84].

Membrane stretch or increases in transmural pressure cause contraction of vascular smooth muscle cells, *ie*, myogenic response[85]. Early studies revealed that the myogenic response was associated with membrane depolarization[86]. Ion channels sensitive to mechanical stimuli have been suggested to serve as the sensor element of the myogenic response of vascular smooth muscle. Mechano-sensitive Cl^- channels and VRCCs have been observed in vascular smooth muscle cells[7, 87] and a pressure-induced Cl^- efflux was reported[88]. Activation of VRCCs and ClC-3 has been postulated to participate in the myogenic response[7, 84, 86, 87], such as in the membrane depolarization and contraction mediated by activation of α_1-adrenoceptors and vascular wall distension due to increased transmural pressure[89]. However, convincing functional evidence for the functional role of VRCCs or ClC-3 in myogenic response and myogenic tone is still lacking due to the lack of specific Cl^- channel blockers[90]. Further study using the ClC-3 knockout or transgenic mice may provide more insights into the functional role of ClC-3 and VRCCs in the regulation of myogenic response to mechanical stretch.

Arterial VSMC proliferation is a key event in the development of hypertension-associated vascular disease[83]. Recent accumulating evidence suggests an important role of ClC-3 and VRCCs in the regulation of cell proliferation induced by numerous mitogenic factors[61]. The magnitude of VRCC currents in actively growing VSMCs is higher than in growth-arrested or differentiated VSMCs, suggesting that VRCCs may be important for VSMC proliferation[91]. Antisense oligonucleotide-mediated downregulation of ClC-3 dramatically inhibits cell proliferation of rat aortic VSMCs[20]. A recent study found that static pressure increased VRCCs and ClC-3 expression and promoted rat aortic VSMC proliferation and cell cycle progression[83]. Inhibition of VRCCs with pharmacological blockers (such as DIDS or the NADPH oxidase inhibitor DPI) or knockdown of ClC-3 with ClC-3 antisense oligonucleotide transfection attenuated pressure evoked cell proliferation and cell cycle progression. Static pressure enhanced the production of ROS in aortic smooth muscle cells. DPI or apocynin pretreatment inhibited pressure-induced ROS production as well as cell proliferation. Furthermore, DPI or apocynin attenuated the pressure-induced upregulation of ClC-3 protein and VRCC current. These data suggest that VRCCs may play a critical role in static pressure-induced cell proliferation and cell cycle progression. Therefore, VRCCs may be of unique therapeutic importance for treatment of hypertension attendant vascular complications.

Cerebral resistance arteries undergo remodeling of the vascular walls during chronic hypertension, which is caused by the coordination of vascular smooth muscle cell proliferation and migration, endothelial cell dysfunction, inflammation and fibrosis. A very recent study demonstrated that the expression of ClC-3 and VRCC activity were increased in basilar artery during hypertension and simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase widely used in clinics for the treatment of hypercholesterolemia, normalized the upregualtion of ClC-3[92]. Furthermore, simvastatin ameliorated hypertension-caused cerebrovascular remodeling through inhibition of VRCCs and ClC-3 and cell proliferation[92]. These effects of simvastatin were abolished by pretreatment with mevalonate or geranylgeranyl pyrophosphate. In addition, Rho A inhibitor C3 exoenzyme and Rho kinase inhibitor Y-27632 both reduced cell proliferation and activation of VRCCs. ClC-3 overexpression decreased the suppressive effect of simvastatin on endothelin-1 and hypoosmolarity-induced cell proliferation. These results provided novel mechanistic insight into the beneficial effects of statins in the treatment of hypertension and stroke through an inhibition of ClC-3 and VRCC function.

ClC-3 and superoxide transport and interaction with NADPH oxidase

ROS has been implicated in cellular signaling processes as well as a cause of oxidative stress-induced cell proliferation[93]. One of the major sources of ROS in the heart and vasculature is through one or more isoforms of the phagocytic enzyme NADPH oxidase, a membrane-localized protein which generates the superoxide ($O_2^{\cdot-}$) anion on the extracellular surface of the plasma membrane (Figure 1). As a charged and short lived anion, it is believed that $O_2^{\cdot-}$ flux is insufficient to initiate intracellular signaling due to the combination of poor permeability through the phospholipid bilayer[94] and a rapid dismutation to its uncharged and more stable derivative, hydrogen peroxide[95, 96]. However, recent evidence has indicated discrete signaling roles for both O_2 and H_2O_2[97].

In response to monocrotaline-induced pulmonary hypertension the expression of *ClCn3* gene was upregulated in rat pulmonary artery[98]. In canine cultured pulmonary arterial smooth muscle cells (PASMCs) incubated with inflammatory mediators *Clcn3* gene was also upregulated[98]. Overexpression of ClC-3 in PASMCs enhanced viability of the cells against H_2O_2, thus suggesting that ClC-3 may improve the resistance of VSMCs to ROS in an environment of elevated inflammatory cytokines in hypertensive pulmonary arteries[98]. It was found that extracellular $O_2^{\cdot-}$, but not H_2O_2, led to Ca^{2+} signaling and apoptosis in pulmonary endothelial cells[99]. This indicates that extracellular $O_2^{\cdot-}$ produced by NADPH oxidase or other sources either crosses the plasma membrane or modifies cell surface proteins to mediate cell signaling (Figure 1).

Recently, Hawkins *et al* studied the transmembrane flux of $O_2^{\cdot-}$ in pulmonary microvascular endothelial cells[17]. Application of an extracellularbolus of $O_2^{\cdot-}$ resulted in rapid and concentration-dependent transient $O_2^{\cdot-}$-sensitive fluorophore hydroethidine (HE) oxidation that was followed by a progressive and nonreversible increase in nuclear HE fluorescence. These fluorescence changes were inhibited by superoxide dismutase (SOD), and the Cl^- channel blocker DIDS, and selective silencing of ClC-3 by treatment with siRNA. Extracellular $O_2^{\cdot-}$ triggered Ca^{2+} release, in turn triggered mitochondrial membrane potential alterations that were followed by mitochondrial $O_2^{\cdot-}$ production and cellular apoptosis. These “signaling” effects of $O_2^{\cdot-}$ were prevented by DIDS, by depletion of intracellular Ca^{2+} stores with thapsigargin and by chelation of intracellular Ca^{2+}. This study demonstrates that $O_2^{\cdot-}$ flux across the endothelial cell plasma membrane occurs through ClC-3 channels and induces intracellular Ca^{2+} release, which activates mitochondrial $O_2^{\cdot-}$ generation. These and other studies suggest that activation of ClC-3 may indeed play a role in cell proliferation, growth, volume regulation and apoptosis of VSMCs.

Conclusion

Regulation of ClC-3 functions in the cardiovascular system is emerging as a novel and important mechanism for the electrical and structural remodeling of the heart and vasculature. However, the integrated function of ClC-3 as a key component of VRCC and Nox1 and as a transport of superoxide needs to be further explored. Although specific gene targeting and transgenic approaches have been proven very powerful for specifically addressing the questions, it will be ideal if specific compounds for ClC-3 can be developed as pharmacological tools to answer these questions and to develop drugs targeting ClC-3 as novel therapeutic tools for the treatment of many cardiac and vascular diseases such as myocardial hypertrophy, ischemia, heart failure, and hypertension.

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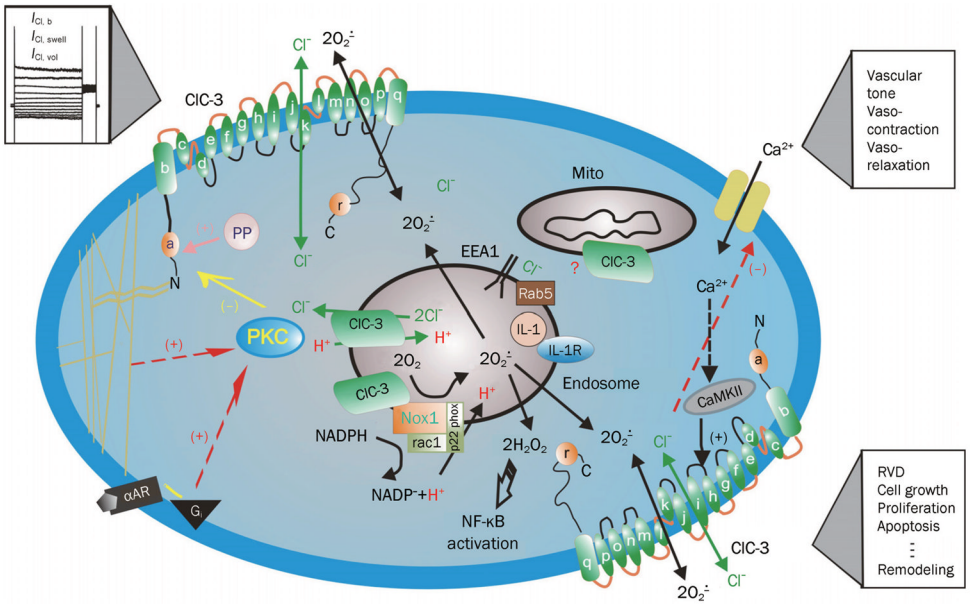
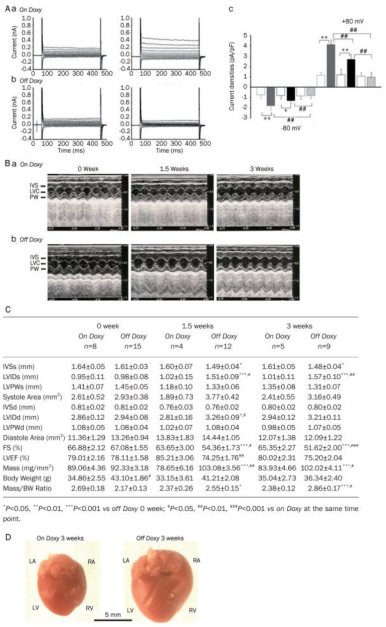


Figure 1.

Schematic representation of regulation and function of ClC-3 Cl^- channels in cardiac myocytes and vascular smooth muscle cells. ClC-3, a member of voltage-gated ClC Cl^- channel family, encodes Cl^- channels in cardiac myocytes and vascular smooth muscle cells that are volume regulated ($I_{Cl,\ vol}$) and can be activated by cell swelling ($I_{Cl,\ swell}$) induced by exposure to hypotonic extracellular solutions or possibly membrane stretch. $I_{Cl,b}$ is a basally activated ClC-3 Cl^- current. Membrane topology model (α-helices a–r) for ClC-3 is modified from Dutzler *et al*[100]. ClC-3 proteins are expressed on both sarcolemmal membrane and intracellular organelles including mitochondria (mito) and endosomes. The proposed model of endosome ion flux and function of Nox1 and ClC-3 in the signaling endosome is modified from Miller Jr *et al*[101]. Binding of IL-1β or TNF-α to the cell membrane initiates endocytosis and formation of an early endosome (EEA1 and Rab5), which also contains NADPH oxidase subunits Nox1 and p22phox, in addition to ClC-3. Nox1 is electrogenic, moving electrons from intracellular NADPH through a redox chain within the enzyme into the endosome to reduce oxygen to superoxide. ClC-3 functions as a chloride-proton exchanger, required for charge neutralization of the electron flow generated by Nox1. The ROS generated by Nox1 result in NF-κB activation. Both ClC-3 and Nox1 are necessary for generation of endosomal ROS and subsequent NF-κB activation by IL-1β or TNF-α in VSMCs. PKC, protein kinase C; PP, serine-threonine protein phosphatases; α-AR, α-adrenergic receptor; G_i, heterodimeric inhibitory G protein; Nox: NADPH oxidase; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; (+) stimulation; (−) inhibition.



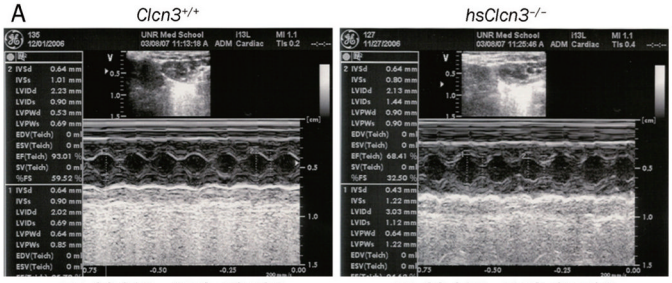
	0 week		1.5 weeks		3 weeks	
	On Doxy *n*=8	*Off Doxy* *n*=15	*On Doxy* *n*=4	*Off Doxy* *n*=12	*On Doxy* *n*=5	*Off Doxy* *n*=9
IVSs (mm)	1.64±0.05	1.61±0.03	1.60±0.07	1.49±0.04*	1.61±0.05	1.48±0.04*
LVIDs (mm)	0.95±0.11	0.98±0.08	1.02±0.15	1.51±0.09$^{***,\#}$	1.01±0.11	1.57±0.10$^{***,\#\#}$
LVPWs (mm)	1.41±0.07	1.45±0.05	1.18±0.10	1.33±0.06	1.35±0.08	1.31±0.07
Systole Area (mm^2)	2.61±0.52	2.93±0.38	1.89±0.73	3.77±0.42	2.41±0.55	3.16±0.49
IVSd (mm)	0.81±0.02	0.81±0.02	0.76±0.03	0.76±0.02	0.80±0.02	0.80±0.02
LVIDd (mm)	2.86±0.12	2.94±0.08	2.81±0.16	3.26±0.09**	2.94±0.12	3.21±0.11
LVPWd (mm)	1.08±0.05	1.08±0.04	1.02±0.07	1.08±0.04	0.98±0.05	1.07±0.05
Diastole Area (mm^2)	11.36±1.29	13.26±0.94	13.83±1.83	14.44±1.05	12.07±1.38	12.09±1.22
FS (%)	66.88±2.12	67.08±1.55	63.65±3.00	54.36±1.73$^{***,\#}$	65.35±2.27	51.62±2.00$^{***,\#\#}$
LVEF (%)	79.01±2.16	78.11±1.58	85.21±3.06	74.25±1.76$^{\#\#}$	80.02±2.31	75.20±2.04
Mass (mg/mm^2)	89.06±4.36	92.33±3.18	78.65±6.16	103.08±3.56$^{***,\#\#}$	83.93±4.66	102.02±4.11$^{***,\#}$
Body Weight (g)	34.86±2.55	43.10±1.86$^{\#}$	33.15±3.61	41.21±2.08	35.04±2.73	36.34±2.40
Mass/BW Ratio	2.69±0.18	2.17±0.13	2.37±0.26	2.55±0.15*	2.38±0.12	2.86±0.17$^{***,\#}$

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs off Doxy* 0 week; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs on Doxy* at the same time point.

Figure 2.

Effects of inducible heart-specific ClC-3 knockout on cardiac volume-regulated Cl^- current (VRCC) and heart function. (A) Representative current traces in isotonic condition and under hypotonic challenge recorded in freshly isolated atrial myocytes from the inducible heart-specific ClC-3 knockout (*doxyhsClC-3*$^{-/-}$) mice with doxycycline (*on Doxy*) in the diet (panel a), or after withdraw of doxycycline (*off Doxy*) from the diet for 3 weeks (panel b). (c) Summary of VRCC current densities in isotonic and hypotonic solutions, recorded at +80 mV and −80 mV. Open boxes, under isotonic conditions; filled boxes under hypotonic conditions; Grey boxes, on doxycycline; black boxes, off doxycycline 1.5 weeks; pale grey boxes, off doxycycline for 3 weeks. $^{**}P<0.01$, hypotonic-induced VSOAC current densities compared to isotonic conditions. $^{\#\#}P<0.01$, hypotonic-induced VSOAC current densities compared between *on Doxy* and 1.5 weeks *off Doxy*, and between 1.5 and 3 weeks *off Doxy* using ANOVA. (B) Representative M-mode echocardiography from *on Doxy* (a) and *off Doxy* (b) mice. (C) Time-dependent changes in M-mode echocardiogram of age-matched *on Doxy* or *off Doxy* for 1.5 and 3 weeks. (D) Comparison of hearts isolated from age-matched (11-week old) *doxyhsClcn3*$^{-/-}$ mice *on Doxy* or *off Doxy* for 3 weeks. Hearts were cleaned up blood and connective tissues and fixed in 4% paraformaldehyde. (Adapted from Xiong *et al*[50]).

A



B

	Clcn3$^{+/+}$ (*n*=8)	*hsClcn3*$^{-/-}$ (*n*=8)
IVSd (mm)	0.54±0.02	0.56±0.02
IVSs (mm)	1.31±0.09	1.01±0.04*
IVIDd (mm)	2.63±0.16	2.69±0.09
LVIDs (mm)	0.84±0.07	1.52±0.09***
LVPWd (mm)	0.74±0.05	0.81±0.05
LVPWs (mm)	1.22±0.05	1.23±0.05
LVEF	0.97±0.04	0.80±0.03***
% FS	67.05±3.57	43.88±2.85***
HR (bpm)	495.71±22.58	396.13±19.11**
LVM (mg)	40.58±4.33	52.22±2.91*

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* *Clcn3*$^{+/+}$.

C

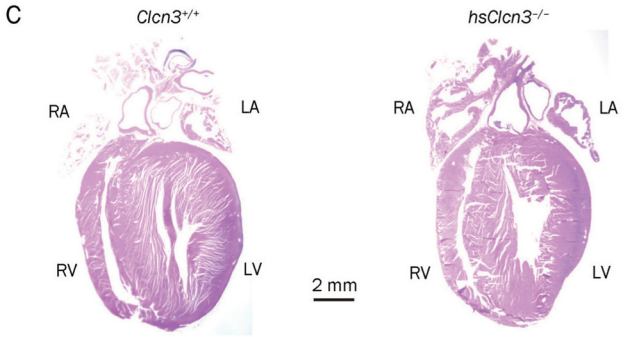


Figure 3.

Echocardiography of cardiac function of wild type and heart-specific ClC-3 knockout mice. (A) Representative M-mode echocardiography from wild-type (*Clcn3*$^{+/+}$; left) and heart-specific ClC-3 knockout (*hsClcn3*$^{-/-}$; right) mice. (B) Echocardiographic measurements in *Clcn3*$^{+/+}$ and *hsClcn3*$^{-/-}$ mice. IVSd, interventricular septum thickness at the end of diastole; LVIDd, left ventricular (LV) dimension at the end of diastole; LVPWd, LV posterior wall thickness at the end of diastole; IVSs, interventricular septum thickness at the end of systole; LVIDs, LV dimension at the end of systole; LVPWs, LV posterior wall thickness at the end of systole; LVEP, calculated LV ejection fraction; %FS, LV fractional shortening; Estimated LV mass, LVM (mg)=1.05[(IVS+LVID+LVPW)^3−(LVID)^3], where 1.05 is the specific gravity of the myocardium. (C) Single longitudinal section (8 μm) of hearts to demonstrate all four heart chambers. Longitudinal were stained with hematoxylin and eosin (Bar=2 mm) (Ye L and Duan DD. unpublished data).