Chloride channels in stroke

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Vascular remodeling of cerebral arterioles, including proliferation, migration, and apoptosis of vascular smooth muscle cells (VSMCs), is the major cause of changes in the cross-sectional area and diameter of the arteries and sudden interruption of blood flow or hemorrhage in the brain, i.e., stroke. Accumulating evidence strongly supports an important role for chloride (Cl\(^{-}\)) channels in vascular remodeling and stroke. At least three Cl\(^{-}\) channel genes are expressed in VSMCs: 1) the TMEM16A (or Ano1), which may encode the calcium-activated Cl\(^{-}\) channels (CACCs); 2) the CLC-3 Cl\(^{-}\) channel and Cl\(^{-}/H^{+}\) antiporter, which is closely related to the volume-regulated Cl\(^{-}\) channels (VRCCs); and 3) the cystic fibrosis transmembrane conductance regulator (CFTR), which encodes the PKA- and PKC-activated Cl\(^{-}\) channels. Activation of the CACCs by agonist-induced increase in intracellular Ca\(^{2+}\) causes membrane depolarization, vasoconstriction, and inhibition of VSMC proliferation. Activation of VRCCs by cell volume increase or membrane stretch promotes the production of reactive oxygen species, induces proliferation and inhibits apoptosis of VSMCs. Activation of CFTR inhibits oxidative stress and may prevent the development of hypertension. In addition, Cl\(^{-}\) current mediated by gamma-aminobutyric acid (GABA) receptor has also been implicated a role in ischemic neuron death. This review focuses on the functional roles of Cl\(^{-}\) channels in the development of stroke and provides a perspective on the future directions for research and the potential to develop Cl\(^{-}\) channels as new targets for the prevention and treatment of stroke.

Keywords: chloride channel; stroke; hypertension; vascular remodeling; oxidative stress

Introduction

Stroke, or cerebrovascular accident, is the second leading cause of death and a foremost cause of serious, long-term disability in the world. It represents a major economic burden with considerable public health impact. A stroke is defined as a rapid loss of brain function(s) due to neuron death and tissue infarction in brain caused by blockage or rupture of blood vessel\(^{[1]}\). Depending on the extent and location of damage in brain, stroke could differently affect physical and mental functions of human being\(^{[2,3]}\). There are two types of stroke: ischemic stroke and hemorrhagic stroke. The former is more prevalent than the latter, comprising approximately 87% of all stroke cases. The risk factors of stroke mainly include old age, hypertension, atrial fibrillation, dyslipidemia, diabetes, atherosclerosis, carotid stenosis, and previous stroke\(^{[4]}\). Hypertension is the most important risk factor of stroke. A sudden increase in blood pressure can make vessel rupture and result in hemorrhagic stroke\(^{[5]}\). The pathophysiological process of stroke refers to cellular and vascular mechanisms. Vascular remodeling of cerebral arterioles, including proliferation, migration, and apoptosis of vascular smooth muscle cells (VSMCs), is the major cause of changes in the cross-sectional area and diameter of the arteries and sudden interruption of blood flow or hemorrhage in the brain\(^{[6-8]}\). Oxidative stress due to excessive production of reactive oxygen species (ROS) promotes VSMC proliferation, migration and apoptosis, matrix remodeling, and neointimal hyperplasia in vascular structure\(^{[9]}\). Meanwhile, oxidative stress results in endothelial dysfunction which is an important mechanism of cerebrovascular damage, or stroke\(^{[10,11]}\).

Recent studies have accumulated compelling evidence that Cl\(^{-}\) channels are closely associated with some risk factors of stroke and may play important roles in the pathophysiological mechanism of vascular remodeling. At least three Cl\(^{-}\) channel genes are expressed in VSMCs: 1) the TMEM16A (anocytamin 1 or Ano1), which may encode the calcium-activated Cl\(^{-}\) channels (CACCs)\(^{[12-14]}\); 2) the CLC-3 Cl\(^{-}\) channel and Cl\(^{-}/H^{+}\) antiporter, which is closely related to the volume-regulated Cl\(^{-}\) channels (VRCCs)\(^{[15-21]}\); and 3) the cystic fibrosis transmembrane conductance regulator (CFTR), which encodes the PKA- and PKC-activated Cl\(^{-}\) channels. Activation of the CACCs by agonist-induced increase in intracellular Ca\(^{2+}\) causes membrane depolarization, vasoconstriction, and inhibition of VSMC proliferation. Activation of VRCCs by cell volume increase or membrane stretch promotes the production of reactive oxygen species, induces proliferation and inhibits apoptosis of VSMCs. Activation of CFTR inhibits oxidative stress and may prevent the development of hypertension. In addition, Cl\(^{-}\) current mediated by gamma-aminobutyric acid (GABA) receptor has also been implicated a role in ischemic neuron death. This review focuses on the functional roles of Cl\(^{-}\) channels in the development of stroke and provides a perspective on the future directions for research and the potential to develop Cl\(^{-}\) channels as new targets for the prevention and treatment of stroke.
membrane conductance regulator (CFTR), which encodes the protein kinase A (PKA)- and protein kinase C (PKC)-activated Cl– channels[22–24]. These Cl– channels are involved in the regulation of many cellular functions of the VSMCs, including the membrane potentials, vascular tone, cell proliferation, migration, and apoptosis (Figure 1)[17, 18, 23, 25, 26]. They are linked to hypertension in many ways. For example, some new studies showed that in hypertensive animal models the expression of VRCCs was increased in VSMCs and activation of VRCCs promoted proliferation and inhibited apoptosis of VSMCs[17, 27], whereas the expression of CACCs was decreased in VSMCs and activation of CACCs was a negative regulator for proliferation of VSMCs[14]. Atherosclerosis, a chronic inflammatory disease[28], also resulted in cerebrovascular remodeling in which proliferation of VSMCs was fundamental[29, 30]. The latest study reported VRCC attended atherosclerotic plaque

Figure 1. Cl– channels and proposed functions in vascular smooth muscle cells. Cl– channels and their corresponding molecular candidate genes and cellular functions are indicated. CFTR, cystic fibrosis transmembrane conductance regulator, encodes Cl– channels activated by stimulation of cAMP-protein kinase A (PKA) pathway (I_{c({\text{Cl,PKA}})}), protein kinase C (PKC) (I_{c(\text{Cl,PKC})}), or extracellular ATP through purinergic receptors (I_{c(\text{Cl,ATP})}). CFTR is composed by two membrane spanning domains (MSD1 and MSD2), two nucleotide binding domains (NBD1 and NBD2) and a regulatory subunit (R). Gi, heterodimeric inhibitory G protein; A1R, adenosine type 1 receptor; AC, adenylyl cyclase; H2R, histamine type II receptor; Gs, heterodimeric stimulatory G protein; α-AR, α-adrenergic receptor; β-AR, β-adrenergic receptor; P2R, purinergic type 2 receptor; P, phosphorylation sites for PKA and PKC; PP, serine-threonine protein phosphatases. I_{c(\text{Cl,Ca})} is a Cl– current activated by increased intracellular Ca2+ concentration ([Ca2+]i); Molecular candidates for I_{c(\text{Cl,Ca})} include TMEM16A (transmembrane protein 16A), the Bestrophin gene family and CLCA1, a member of a Ca2+-sensitive Cl– channel family (CLCA). CLC-3, a member of voltage-gated CLC Cl– channel family, encodes Cl– channels that are volume-regulated (I_{c(\text{Cl,vol})}) and can be activated by cell swelling (I_{c(\text{Cl,swell})}) induced by exposure to hypotonic extracellular solutions or possibly membrane stretch. I_{c(\text{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[85]. 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 adapted from Miller Jr et al[86]. 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. Statins block CLC-3 channels, which causes hyperpolarization of the cell membrane, closure of Ca2+ channels and vasorelaxation, and inhibition of cell proliferation. Nox: NADPH oxidase.
formation. VRCC may also play important roles in ischemic neuron apoptosis. In addition, gamma-aminobutyric acid (GABA) receptor-mediated Cl– current has also been implicated a role in ischemic neuron death. This review focuses on the functional role of Cl– channels in stroke and provides a perspective on the future directions for research and the potential to develop Cl– channels as novel therapeutic targets for the prevention and treatment of stroke.

**Calcium-activated Cl– channels (CACCs) and stroke**

The Cl– current evoked by a rise in intracellular Ca2+ concentration ([Ca2+]i) and free Ca2+, or Ca2+-activated Cl– current (ICl, Ca), was first described in *Xenopus* oocytes in 1982[31, 32]. Later studies found that similar ICl, Ca is present widely in various cell types, such as endothelial cells and VSMCs of different organs, of many species, including human[33–35]. In the last two decades CACCs in VSMCs have received extensive attention due to the important roles of CACCs in multiple cellular functions of VSMCs, ranging from the regulation of the resting membrane potential (RMP), intracellular Ca2+ ([Ca2+]i), and vascular tone to the control of cell proliferation and apoptosis[25, 35-37]. But, the molecular identity of CACCs in VSMCs has not been fully defined[38, 39]. Several genes, including CLCA, Tweety, and bestrophin, have been proposed to encode the pore forming channel protein of CACCs[40-43]. Using siRNA technique, however, Wang et al excluded the contribution of bestrophin 3 to ICl, Ca in rat basilar artery smooth muscle cells (BASMCs)[14]. Recent independent studies from three laboratories have provided compelling evidence that the TMEM16/Anoctamin proteins, a family of transmembrane proteins, may be the pore forming channel protein of CACCs[44-46]. Heterogenous expression of TMEM16A/Ano1 or TMEM16B/Ano2 produced a Cl– current with characteristics, including Ca2+ and voltage-dependence, outward rectification and ion selectivity, similar to the endogenous ICl, Ca in many types of native cells[12, 44-49]. Thomas-Gatewood et al have found that TMEM16A channel proteins are expressed and inserted into the plasma membrane of rat cerebral arterial smooth muscle cells (CASMCS)[13]. Whole-cell ICl, Ca in the CASMCS displayed properties similar to those generated by rTMEM16A channels, including the Ca2+-dependent activation, current-voltage (I–V) relationship linearization by an elevation in [Ca2+]i, and an I–Ca permeability sequence. A pore-targeting TMEM16A antibody that effectively blocked rTMEM16A currents also inhibited ICl, Ca in the cerebral arterial SMCs. TMEM16A knockdown using small interfering RNA (siRNA) also inhibited ICl, Ca in the CASMCS. These data strongly support the notion that ICl, Ca in the CASMCS is encoded by TMEM16A. More recently, Wang et al have found that TMEM16A, TMEM16C, TMEM16E-F, and TMEM16K are expressed at high levels endogenously in rat BASMCs, while TMEM16B and TMEM16D were not detected in these cells. Knockdown of TMEM16A with siRNA remarkably attenuated endogenous ICl, Ca in BASMCs, suggesting that TMEM16A encodes the CACCs in BASMCs[14].

Several studies have provided strong evidence for a key role of CACCs in the stroke-related vascular remodeling. In BASMCs isolated from 2k2c hypertensive rats, the expression of TMEM16A and functional ICl, Ca was significantly reduced. The activity of ICl, Ca negatively correlated with the blood pressure and the medial cross sectional area of basilar artery during the development of hypertension[14]. ICl, Ca began to drop within 1 week after 2k2c operation and the expression of TMEM16A was reduced to a significant level at week 4 after surgery. It is currently not clear why down-regulation of TMEM16A expression lagged behind the decreased activity of CACCs. Wang et al found that increased activity of CaMK II inhibited ICl, Ca and CaMK II activity was up-regulated at week 1 after 2k2c surgery. Therefore, the increased CaMK II activity in the 2K2C rats may be one of the major molecular mechanisms for the down-regulation of functional CACCs in the hypertensive rat[14]. Wang et al further demonstrated CACC is a negative regulator of cell proliferation of BASMCs. TMEM16A-mediated CACCs inhibited cell proliferation by arresting the cell cycle at G0/G1 phase through reduction of cyclin D1 and cyclin E expression. Therefore, down-regulation of CACCs in VSMCs may play an important role in hypertension-induced structural remodeling of cerebral arteries[14]. It has been demonstrated that CACCs may be a critical regulator of cell proliferation in Ehrlich lette ascite cells[50]. The important role of Caccs and TMEM16A in the regulation of proliferation suggests that CACCs may be new molecular targets for the prevention and treatment of hypertension-induced vascular remodeling and stroke.

**Volume-regulated Cl– channels (VRCCs) and stroke**

VRCCs are ubiquitously distributed in mammalian cells including neurons, VSMCs, and endothelial cells. VRCCs are involved in many pathophysiological functions such as cell volume regulation, proliferation, differentiation and apoptosis. Although recent studies on the molecular identity of VRCCs are inconsistent, CLC-3, a member of voltage-gated CLC Cl– channel family, is thought to be responsible for VRCCs and mediate volume regulation in many cell types[16, 51-53]. A study in A10 VSMCs strongly supported that CLC-3 was the molecular component responsible for the activation and regulation of VRCCs[54]. Although CLC-2 channels, another member of the CLC family, are also volume-regulated Cl– channels and involved in cell volume regulation[55] they differ significantly from native VRCCs in voltage sensitivity, anion selectivity, and pharmacology[56].

Several recent studies suggest that VRCCs and CLC-3 are closely associated with blood pressure regulation and may play an important role in hypertension-induced cerebrovascular remodeling[15, 20, 21, 27, 37]. Shi et al found that functional VRCCs and CLC-3 expression were increased in hypertensive rat BASMCs and the increment of CLC-3 expression was correlated with the severity of hypertension, suggesting that VRCCs and CLC-3 were involved in vascular remodeling during chronic hypertension[27]. Later study from Qian et al found that static pressure increased VRCCs and CLC-3 expression in rat aortic SMCs, suggesting that elevation in blood pressure may directly up-regulate the expression of CLC-3 in
Inhibition of VRCCs with pharmacological blockers or knockdown of CLC-3 with CLC-3 antisense oligonucleotide dramatically inhibited the static pressure-induced cell proliferation and cell cycle progression of rat aortic SMCs. VRCC currents in actively growing VSMCs were higher than that in growth-arrested or differentiated SMCs, suggesting that VRCCs may be important for SMC proliferation. Silencing endogenous CLC-3 by antisense oligonucleotide significantly inhibited endothelin-1 (ET-1) induced cell proliferation in BASMCs, which is consistent with previous studies in rat aortic SMCs. CLC-3 contributed to phosphorylation of the Akt and glycogen synthase kinase-3β (GSK-3β), resulting in up-regulation of cyclin D1 and cyclin E which pushed forward cell cycle from G1 to S phase, whereas silencing CLC-3 protein effectively up-regulated cyclin-dependent kinase inhibitors (CDKIs) p27KIP and p21CIP and inhibited cell cycle.

The beneficial effects of statins on cerebrovascular remodeling during hypertension may be due to the inhibition of CLC-3 via modifying Rho activity and prevent the incidence of ischemic stroke. Chu et al found that CLC-3 was necessary for the activation of SMCs by TNF-α and neointimal hyperplasia. TNF-α induced a NADPH-dependent generation of intracellular reactive ROS and CLC-3 provided charge neutralization for the NADPH oxidase electron current. ERK1/2 activation is linked to ROS production and ERK1/2 is upstream of MMP-9 activation and proliferation. ROS activation of the transcription factor NF-κB is widely recognized as a key regulatory step in vascular inflammation. Therefore, CLC-3 is very critical for TNF-α induced cell proliferation and inflammation response. These findings identify CLC-3 as a novel target for the prevention of inflammatory and proliferative vascular diseases referred in stroke. Generally speaking, cell proliferative regulation is a complex network. When ERK1/2 and MMP-9 induce proliferation of VSMCs, they are likely to regulate cyclin D1 and cyclin E. To date, the molecular mechanisms for CLC-3 regulation of VSMC proliferation is not completely clear and further studies are needed.

The process of cerebrovascular remodeling during hypertension prior to stroke involves not only the increased proliferation but also the decreased apoptosis of VSMCs. In fact, the balance between proliferation and apoptosis is broken. A study showed that overexpression of CLC-3 significantly decreased the apoptotic rate of H2O2-treated BASMCs and increased the cell viability, whereas silencing of CLC-3 produced opposite effects and increased the apoptotic rate. CLC-3 overexpression decreased cytochrome c release and caspase-3 activation, and increased both the stability of mitochondrial membrane potential and the ratio of Bcl-2/Bax. It also inhibited the degradation of cell skeleton protein Lamin induced by H2O2. This report is consistent with previous study in PC12 cells which also demonstrated CLC-3 mediated apoptotic process induced by thapsigargin through inhibitory mechanism. But another study in human prostate cancer epithelial cells reported opposite link between CLC-3 and Bcl-2 which demonstrated Bcl-2 up-regulated CLC-3 expression and increased IC50 of thapsigargin to inhibit apoptosis. Moreover, endothelial progenitor cell and endothelial cell dysfunction are also important for hypertension development. CLC-3 could mediate extracellular O2- to enter endothelial cell to stimulate the production of ROS which damages endothelial cell to increase endothelin-1 release and decrease NO release contributing to hypertension development. Until now very little is known about the roles of VRCCs and CLC-3 in endothelial progenitor cells.

Atherosclerosis is closely associated with vascular remodeling. Atherosclerosis involves the phenotypic changes of several types of cells, especially monocyte-derived macrophages and VSMCs, and the formation of foam cells. Atherosclerotic plaque rupture and cholesterol release can block vessels in brain and cause stroke. One study suggested that volume-regulated Cl- movement was augmented during macrophage-derived foam cell formation, and its increment positively correlated with atherosclerotic plaque area in the development of atherosclerosis. Cell swelling occurred in macrophages due to uptake of modified LDL during the process of foam cell formation and the alteration of Cl- transmembrane movement via VRCCs was involved in the development of atherosclerosis. In contrast, inhibition of VRCCs with Cl- channel blockers prevented ox-LDL induced foam cell formation. But current pharmacological blockers lack specificity for a particular Cl- channel and gene targeting study may give a clearer answer about the contribution of VRCCs to atherosclerosis. Furthermore, SMC-derived foam cell formation in atherosclerosis also refers to an increase in cell volume and VRCC activation. So the role of VRCCs and CLC-3 in SMC-derived foam cell formation warrants further studies in the future.

There is now some evidence that some neurons die as a result of apoptosis after cerebral ischemia. Apoptotic cell volume decrease has also been associated with regulatory volume decrease (RVD) since the decrease of cell volume after apoptotic stimuli is due to activation of the same K+ and Cl- channels that are responsible for physiological RVD. VRCCs play a predominant role in neuronal apoptosis. Cl- channel blockers NPPB, SITS and DIDS completely inhibited cell shrinkage, RVD, caspase-3 activation and DNA laddering in neurons, suggesting Cl- channel blockers had a preventive effect on neuron apoptosis. These blockers abolished structural alterations that occur during cell apoptosis, such as chromatin condensation and leaky nuclear envelopes. But in another study these blockers just mildly attenuated cell death induced by staurosporine, C6-ceramide, or serum deprivation and had no significant effects on caspase-3 activation and DNA fragmentation at concentration that prevented cell shrinkage. It should be pointed out that, however, all these blockers of Cl- channels are not specific. They might produce their effects by blocking other ion channels or regulating other signaling pathways.

Nevertheless, regulation function of VRCCs in the cardiovascular system is emerging as a novel and important mechanism for the structural remodeling of the vasculature and may provide a novel therapeutic approach for the treat-
ment of many vascular diseases, such as hypertension, atherosclerosis and stroke. CLC-3 may be potential new targets for the prevention of the cerebrovascular remodeling that occurs during the development of hypertension.

**CFTR and stroke**

CFTR Cl\(^{-}\) channels are expressed in VSMCs and may play an important role in the regulation of vascular tone. Individuals homozygous for the autosomal recessive disorder cystic fibrosis (CF) are known to have low blood pressure\(^{[79]}\). Older female CF carriers had lower systolic and diastolic pressures than matched healthy subjects, with a tendency for blood pressure to increase less with age\(^{[79]}\). A beneficial effect of CF gene mutation may be protection against developing hypertension and significantly reduce stroke and heart disease in the female CF patients\(^{[79]}\). The effect of activation of CFTR channels on blood pressure is insufficient to prevent hypertension, though it remains conceivable that the severity might be ameliorated in carriers. How CFTR is linked to hypertension and stroke in vivo is currently not known. It was proposed that this might be a result of life-long increased sweat Na\(^{+}\) and Cl\(^{-}\) loss\(^{[78, 80]}\).

**Cl\(^{-}\) current mediated by GABA receptor and stroke**

Stroke therapy has focused on reducing risk factors and minimizing secondary brain damage by restoring and maintaining perfusion. However, a third approach, neuroprotection, is now being investigated. GABA is the primary inhibitory neurotransmitter in mammalian brain and increasing GABA function via activation of GABA\(_{\text{A}}\) receptor resulted in increased Cl\(^{-}\) influx and promoted hyperpolarization of neuron membrane. So, during acute ischemic damage in brain increased Cl\(^{-}\) current mediated by GABA receptor probably plays a beneficial role in stroke. There is good evidence that GABA exerted an inhibitory tone on glutamate-mediated neuronal activity\(^{[83]}\). Meldrum proposed that the excitotoxic process of neuron probably depends on a balance between excitatory and inhibitory mechanisms\(^{[84]}\). Agonists of GABA receptors are likely to be new treatment approach for stroke.

**Perspectives**

Cl\(^{-}\) channels are emerging as novel and important mechanisms for stroke and may become novel therapeutic targets for the treatment and prevention of stroke although at present many questions about these channels in stroke are still not clear. Future studies will focus on the clear identification of functional role of each type of Cl\(^{-}\) channels (CFTR, CLC-3, and TMEM16A, etc) in VSMCs, endothelial cells, and endothelial progenitor cells, in cerebrovascular remodeling and VSMC-derived foam cell formation, and neuron apoptosis and necrosis. The findings from these studies may generate new directions for the development of new therapeutic strategies for the treatment of stroke and may reduce the impact of this enormous economic and social burden.

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