Introduction

Stroke or cerebral ischemia is a leading cause of death and long-term disabilities worldwide. Although major advances have occurred in the past decades in the prevention of brain ischemia, treatment is limited to the use of tissue plasminogen activator (tPA), which has limited success and a major side effect of intracranial hemorrhage[1, 2]. Searching for new cell injury mechanisms and therapeutic interventions has become a major challenge in the field. It has been recognized for many years that intracellular Ca²⁺ overload in neurons is essential for neuronal injury associated with brain ischemia. However, the exact pathway(s) underlying the toxic Ca²⁺ loading remained elusive. This review discusses the role of two Ca²⁺-permeable cation channels, TRPM7 and acid-sensing channels, in glutamate-independent Ca²⁺ toxicity associated with brain ischemia.

Keywords: acid-sensing ion channel; TRPM7; brain ischemia; neurons

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Calcium-permeable ion channels involved in glutamate receptor-independent ischemic brain injury

Ming-hua LI¹, Koichi INOUE², Hong-fang SI³, Zhi-gang XIONG⁴, *

¹Department of Psychology, Washington State University, Vancouver, WA, USA; ²Department of Physiology, Hamamatsu University School of Medicine, Hamamatsu Shizuoka 431–3192, Japan; ³School of Pharmacy, Anhui Medical University, Hefei 230032, China; ⁴Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA 30310, USA

Brain ischemia is a leading cause of death and long-term disabilities worldwide. Unfortunately, current treatment is limited to thrombolysis, which has limited success and a potential side effect of intracerebral hemorrhage. Searching for new cell injury mechanisms and therapeutic interventions has become a major challenge in the field. It has been recognized for many years that intracellular Ca²⁺ overload in neurons is essential for neuronal injury associated with brain ischemia. However, the exact pathway(s) underlying the toxic Ca²⁺ loading remained elusive. This review discusses the role of two Ca²⁺-permeable cation channels, TRPM7 and acid-sensing channels, in glutamate-independent Ca²⁺ toxicity associated with brain ischemia.

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Glutamate mediated Ca²⁺-toxicity

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS)[12–14]. Its receptors are widely expressed at soma and dendrites of the CNS neurons. Activation of these receptors is involved in a variety of physiological functions of neurons including synaptic transmission/plasticity, learning/memory, neuronal development and differentiation[12, 13]. Glutamate receptors are classified into two major categories: ionotropic receptors, which are ligand-gated cation channels; and metabotropic receptors, which are coupled through G proteins to second messenger systems[14].
One subtype of ionotropic glutamate receptors, the N-methyl-D-aspartate (or NMDA) receptor, is highly permeable to Ca\(^{2+}\) ions. Activation of these receptors has been considered to play a critical role in Ca\(^{2+}\) toxicity associated with ischemic brain injury\(^{[3, 17-20]}\). Accordingly, blocking these receptors has been shown to be neuroprotective in cell culture and animal models of brain ischemia. Unfortunately, none of the human trials using the antagonists of glutamate receptors showed a satisfactory protection for stroke patients. Although multiple factors, including difficulty in early initiation of treatment and intolerance of severe side effects, may have contributed to the failure of the trials\(^{[4, 21-24]}\), recent studies suggest that Ca\(^{2+}\) entry through glutamate-independent pathways, e.g. TRPM7 channels and Ca\(^{2+}\)-permeable acid-sensing ion channels (ASICs), may contribute to the injury of neurons associated with brain ischemia.

**TRPM channels and ischemic neuronal injury**

Transient receptor potential (TRP) channels belong to a novel family of cation channels that are highly expressed in various tissues including the brain\(^{[25, 26]}\). Several members of TRP family can be activated by oxidative stress and oxygen free radicals, both of which play important roles in neuronal injury associated with stroke or ischemia. Recent work has indicated that members of the melastatin subfamily (TRPM) of the TRP channels, particularly the TRPM7, play a key role in neuronal cell death associated with brain ischemia\(^{[27-31]}\).

The TRP superfamily is a diverse group of voltage-independent calcium-permeable cation channels expressed in mammalian cells\(^{[25, 26]}\). These channels have been divided into six subfamilies, and two of them, TRPC and TRPM, have members that are widely expressed and activated by oxidative stress. TRPC3 and TRPC4 are activated by oxidants, which induce Na\(^{+}\) and Ca\(^{2+}\) entry into cells through phospholipase C-dependent mechanisms. TRPM2 is activated by oxidative stress or TNFalpha, and the mechanism involves production of ADP-ribose, which binds to an ADP-ribose binding cleft in the TRPM2 C-terminus. Treatment of neurons or HEK 293T cells expressing TRPM2 with H\(_2\)O\(_2\) resulted in Ca\(^{2+}\) influx and increased susceptibility to cell death\(^{[27]}\). Inhibition of endogenous TRPM2 function, in contrast, protected cell viability\(^{[27, 30]}\). Nevertheless, the exact role of TRPM2 in Ca\(^{2+}\) toxicity associated with ischemic brain injury remains to be explored.

The potential role of TRPM7 channels in ischemic neuronal death has been described recently\(^{[30, 31]}\). Aarts and colleagues first examined the mechanism of neuronal cell death in ischemic conditions in the presence of glutamate antagonists. Cultured mouse cortical neurons were exposed to oxygen-glucose deprivation (OGD), an in vitro model of ischemia reported to mediate neuronal death through NMDA receptor activation\(^{[33, 34]}\). Blocking the glutamate excitotoxicity in these cultures, however, unmasked a potent, previously unappreciated mechanism of non-excitotoxic neuronal cell death, which became increasingly responsible for neurodegeneration as the duration of OGD was prolonged\(^{[30]}\). Further studies demonstrated that the mechanism of cell death involved activation of a non-selective cation current with high permeability to Ca\(^{2+}\). The current showed outward rectifying properties, was potentiated by reactive oxygen/nitrogen species (ROS), and was blocked by Gd\(^{3+}\). The electrophysiological characteristics and pharmacological properties of the current suggested the involvement of TRPM7 channels. Indeed, molecular biological approaches (e.g. siRNA) confirmed the involvement of TRPM7 channels in glutamate-independent anoxic neuronal injury\(^{[30]}\). Although a specific agonist remains to be determined, these studies suggest that, in ischemic conditions, TRPM7 channels could be activated by ROS. Ca\(^{2+}\) entry through these channels participates in neuronal injury. A lethal positive feedback loop is established when Ca\(^{2+}\) entry through TRPM7 channels stimulates additional ROS production, causing further TRPM7 activation\(^{[30]}\). Blocking TRPM7 channels or suppressing its expression by RNA interference was effective in preventing the death of neurons by OGD.

Very recent studies by Sun and colleagues also demonstrated involvement of TRPM7 channels in the injury of hippocampal neurons in vitro in rat model of global ischemia\(^{[30]}\). Suppressing TRPM7 expression in CA1 neurons by intrahippocampal injections of viral vectors bearing shRNA specific for TRPM7 channels had no ill effect on animal survival, neuronal and dendritic morphology, neuronal excitability, or synaptic plasticity. However, TRPM7 suppression made neurons resistant to ischemic injury and preserved neuronal morphology and function. Also, it prevented ischemia-induced deficits in long-term potentiation and preserved performance in fear-associated and spatial-navigational memory tasks. Thus, regional suppression of TRPM7 is feasible, well tolerated and inhibits delayed neuronal death in vivo. In addition to Ca\(^{2+}\) toxicity mediated by TRPM7 channels, studies by Inoue and colleagues have suggested that Zn\(^{2+}\) permeability of these channels also plays a role in ischemic brain injury\(^{[35]}\).

**Acid-sensing ion channels and ischemic brain injury**

In acute neurological conditions such as brain ischemia, marked reduction of tissue pH takes place\(^{[36-41]}\). Following ischemia, shortage of oxygen supply promotes anaerobic glycolysis, leading to lactic acid accumulation and resultant decrease in brain pH\(^{[42, 43]}\). Increased ATP hydrolysis and release of H\(^{+}\) also contributes to pH drop. At the same time, cessation of local circulation results in carbon dioxide accumulation and carbonic acid build up, which may participate in the decrease of tissue pH\(^{[30]}\). During ischemia, decreases of brain pH to ~ 6.5 are commonly observed. It can also fall to 6.0 or below during severe ischemia or under hyperglycemic conditions\(^{[37, 40, 41, 44, 45]}\).

Decrease of brain pH or acidosis has long been known to play an important role in ischemic brain injury\(^{[30, 42, 43, 46-48]}\). A direct correlation between the degree of brain acidosis and infarct size has also been described\(^{[30, 49]}\). However, the exact mechanism underlying acidosis-mediated neuronal injury remained vague. Acidosis may cause non-selective denaturation of proteins and nucleic acids\(^{[18]}\); trigger cell swelling and osmolysis via stimulation of Na\(^{+}\)/H\(^{+}\) and Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchang-
ers[51]; hinder postischemic metabolic recovery by inhibiting mitochondrial energy metabolism and impairing postischemic blood flow via vascular edema[52]; or stimulate pathologic free radical formation[53]. At the neurotransmitter level, profound acidosis inhibits astrocytic glutamate uptake, which may contribute to excitotoxic neuronal injury.[54]

Interestingly, mild acidosis has been considered to be beneficial in protecting neurons from excitotoxic injury[55–57]. This may be explained by proton inhibition of NMDA channel activity[58, 59]. In contrast to its modulating effect on other ion channels, protons can activate a distinct family of ligand-gated channels, the acid-sensing ion channels (ASICs)[60–71]. ASICs belong to the amiloride-sensitive epithelial Na⁺-channel/sodium channel family. ASICs are permeable to Ca²⁺ ions[61, 82, 83]. ASIC1 and ASIC2 are widely expressed in peripheral sensory neurons[63, 79, 86]. However, ASIC1 and ASIC2 are implicated in the maintenance of retinal integrity[109–113]. In pathological conditions, activation of Ca²⁺-permeable ASIC1 is involved in glutamate-dependent, acidosis mediated, ischemic brain injury[71, 82, 112] and in axon degeneration associated with multiple sclerosis[113]. In contrast, increased expression of ASIC2 is associated with neuronal survival following global ischemia[114], while reduced expression of ASIC1 is associated with neuroprotection elicited by ischemic pre- and post-conditioning[115].

The presence of ASIC1 in the brain, its activation by pH drops to the levels commonly seen during brain ischemia, and its permeability to Ca²⁺ make it a potential player in ischemic brain injury. A series of recent studies, performed in vitro in neuronal cell culture and in vivo in whole animal models of ischemia, have provided strong evidence supporting this hypothesis[71, 82, 112]. In cultured neurons, for example, brief acid incubations induced significant neuronal injury. This acid-induced neuronal injury was glutamate-independent, but was inhibited by amiloride, a non-specific ASIC blocker, or PcTX1, a specific ASIC1 inhibitor. In contrast to the neurons from ASIC1+/− mice, neurons cultured from ASIC1−/− mice were resistant to acid injury. Reducing the concentration of extracellular Ca²⁺, which lowers the driving force for Ca²⁺ entry through ASICs, also decreased acid-induced injury of CNS neurons[71, 82]. Thus, activation of ASICs, and subsequent Ca²⁺ entry, participates in acidosis-mediated injury of neurons.

While homomeric ASIC1 can conduct Ca²⁺, some studies have suggested that, a significant portion of acid-evoked increases of intracellular Ca²⁺ is not due to Ca²⁺ entry directly through ASIC1 homomers. Rather, acidosis might induce Ca²⁺ accumulation through secondary activation of voltage-gated Ca²⁺ channels due to ASIC-mediated membrane depolarization and/or Ca²⁺ release from intracellular stores[114–116].

In vivo studies also support a role for ASIC1a activation in acidosis-mediated, ischemic brain injury[71, 112, 119]. In rats and mice, intracerebral ventricular injection of ASIC1a inhibitors reduced the infarct volume by up to 60%. Similarly, ASIC1a gene knockout protected the mouse brain from ischemic injury. Furthermore, ASIC1a blockade and ASIC1a gene knockout provided additional protection in the presence of glutamate receptor antagonists[71]. The protection by ASIC1a blockade has an effective time window of >5 h, and the protection persists for at least 7 d[119]. Attenuating brain acidosis by intracerebroventricular administration of NaHCO₃ is also protective, further suggesting that acidosis is a mediator of ischemic brain injury.

**Interactions between ASIC1a and hypoxia/ischemia related signals contribute to ischemic brain injury**

In the normal condition, ASIC1a current desensitizes rapidly in the continuous presence of acidosis. This property of ASIC1a argues against its role in brain ischemia in which acidosis is, in general, long-lasting. Recent findings showing that the properties of ASICs, particularly the ASIC1a channel, can be dramatically modulated by ischemia per se and/
or ischemia-related signals have provided good explanation supporting the role of ASIC1a channels in ischemic brain injury\(^{[31, 120-122]}\). In cultured mouse cortical neurons, for example, brief OGD not only increased the amplitude but also reduced the desensitization of the ASIC current. Accordingly, OGD treatment enhanced acidosis-mediated neuronal injury\(^{[31]}\). The cellular and molecular mechanisms underlying ischemia-induced increase of ASIC activity has been investigated extensively by several studies. Allen and Attwell demonstrated that arachidonic acid, a lipid metabolite released in ischemia, increased the amplitude of the ASIC current in rat cerebellar Purkinje neurons\(^{[122]}\). Gao and colleagues demonstrated that an increased phosphorylation of ASIC1a channels by CaMKII, mediated by NMDA receptor activation, was involved in ischemia-induced enhancement of the ASIC responses\(^{[112]}\). Sherwood and Askwith demonstrated that dynorphins, the most basic neuropeptides abundantly expressed in the central nervous system, could increase the activities of ASIC1a channels and enhance neuronal damage following ischemia\(^{[31, 129]}\). They do so by reducing the steady-state desensitization of the ASIC1a channels. Very recent studies by Duan and colleagues also showed that, spermine, one of the endogenous polyamines, exacerbated ischemic neuronal injury through sensitization of ASIC1a channels to extracellular acidicosis\(^{[121]}\). Spermine slows down the desensitization of these channels in the open state, shifting steady-state desensitization to more acidic pH, and accelerating recovery of the channels between repeated periods of acid stimulation. Thus, therapeutic interventions for brain ischemia may target ASICs directly by using ASIC blockers/inhibitors or indirectly by blocking the ischemia-related signals which enhance the activation of ASICs.

**Perspectives**

Stroke/brain ischemia is a leading health problem worldwide. Although in recent years enormous progresses have been made in the prevention of stroke, unfortunately, there is still no effective treatment for stroke patients. Searching for new cell injury mechanisms and effective therapeutic strategies is therefore a major challenge in the field. Brain ischemia initiates various biochemical changes such as increased glutamate release, production of oxygen free radicals, lactic acidosis, and reduced ATP synthesis, etc. These changes may facilitate the opening of various Ca\(^{2+}\)-permeable ion channels such as glutamate-receptor-gated channels, voltage-gated Ca\(^{2+}\) channels, TRPM7 channels, acid-sensing ion channels, etc. Activation of these channels induces entry of Ca\(^{2+}\) and accumulation of intracellular Ca\(^{2+}\). Intracellular Ca\(^{2+}\) accumulation can also occur through other pathways, e.g., release of Ca\(^{2+}\) from intracellular stores, or entry of Ca\(^{2+}\) through reversed Na\(^+\)/Ca\(^{2+}\) exchange system (Figure 1). Overload of neurons with Ca\(^{2+}\) activates a panel of enzymes including proteases, phospholipases and endonucleases, leading to destruction of neurons either through necrotic or apoptotic process. Targeting the pathways responsible for Ca\(^{2+}\) overload may lead to effective neuroprotective interventions for stroke patients. The recent failure of clinical trials using the antagonists of glutamate receptors, however, suggests that future effort should also consider glutamate-independent Ca\(^{2+}\) toxicity in ischemia, e.g., through activation of TRPM7 channels or ASICs.

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