The role of ASICs in cerebral ischemia

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Cerebral ischemia is a leading cause of death and long-term disabilities worldwide. Excessive intracellular Ca²⁺ accumulation in neurons has been considered essential for neuronal injury associated with cerebral ischemia. Although the involvement of glutamate receptors in neuronal Ca²⁺ accumulation and toxicity has been the subject of intensive investigation, inhibitors for these receptors showed little effect in clinical trials. Thus, additional Ca²⁺ toxicity pathway(s) must be involved. Acidosis is a common feature in cerebral ischemia and was known to cause brain injury. The mechanisms were, however, unclear. The finding that ASIC1a channels are highly enriched in brain neurons, their activation by ischemic acidosis, and their demonstrated Ca²⁺ permeability suggested a role for these channels in Ca²⁺ accumulation and neuronal injury associated with cerebral ischemia. Indeed, a number of studies have now provided solid evidence supporting the involvement of ASIC1a channel activation in ischemic brain injury. © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

INTRODUCTION

Ischemic stroke, or cerebral ischemia, is the third most common cause of death in most industrialized countries. Although major advances have occurred in the prevention of stroke during the past several decades, no effective treatment is now available. Current clinical practices for stroke patients utilize thrombolytic agent tissue plasminogen activator (tPA) to reopen the clotted vessels.¹ This approach, however, has very limited success due to a short therapeutic time window of 3 h and side effect of intracranial hemorrhage. On the other hand, cell death is prominent following stroke. Therefore, the need for a continuous search of neuronal damage mechanisms and effective therapeutic strategies for neuroprotection remains high.

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Although multiple pathways and biochemical changes contribute to ischemic brain injury, excessive intracellular Ca²⁺ accumulation and resultant toxicity has been considered essential in the pathology of cerebral ischemia.² In the resting conditions, free intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in neurons is maintained at nanomolar range. Following cerebral ischemia, however, [Ca²⁺]ᵢ can rise to as high as several micromolars. Excessive accumulation of Ca²⁺ in neurons leads to uncontrolled activation of various enzymes causing breakdown of proteins, lipids, and nucleic acids, and the destruction of neurons.³⁻⁵ In addition, overloading Ca²⁺ in mitochondria can cause opening of mitochondria permeability transition pore (PTP), promoting apoptosis through release of cytochrome c and activation of caspases.⁶

Ca²⁺ can enter neurons through various pathways, among which glutamate receptor-gated channels have received the most attention. Unfortunately, clinical trials targeting these channels have shown little effect in improving the outcome of cerebral ischemia.⁷ Multiple factors may have contributed to the failure of the trials. In particular, additional glutamate-independent Ca²⁺ entry and toxicity pathways must be considered.
BRAIN ACIDOSIS IN CEREBRAL ISCHEMIA

Acidosis, a condition characterized by too much acid in the tissue or body fluid, is one of the most common pathophysiological changes in the brain associated with acute neurological conditions such as cerebral ischemia. In the ischemic core, for example, a rapid drop of pH to 6.5 or lower is frequently observed. The lack of oxygen supply promotes anaerobic glycolysis which leads to increased production of lactic acid. Accumulation of lactic acid, along with increased production of H+ from ATP hydrolysis, and release of H+ from presynaptic terminals contributes to the acid buildup in the brain. Acidosis has long been recognized to aggravate brain injury associated with cerebral ischemia. However, the detailed mechanism(s) remained elusive, although a number of possibilities have been suggested long before the role of acid-sensing ion channels (ASICs) was recognized.

ASIC1a ACTIVATION IS INVOLVED IN ACIDOSIS-MEDIATED ISCHEMIC BRAIN INJURY

On the basis of the evidence that ASIC1a subunits are highly expressed in brain neurons, their activation by pH drops to the level commonly seen in cerebral ischemia, and their permeability to Ca2+ and Na+, Xiong and colleagues tested the hypothesis that activation of ASIC1a channels is involved in neuronal Ca2+ accumulation and injury associated with cerebral ischemia. Using patch-clamp recording and fast-perfusion technique, large inward currents were recorded in cultured mouse cortical neurons in response to rapid perfusion of acidic solutions at pH levels relevant to cerebral ischemia. The acid-activated currents in cortical neurons were sensitive to non-specific ASIC blocker amiloride and partially inhibited by ASIC1a-specific inhibitor PcTX1, suggesting that the currents were mediated by ASIC1a-containing channels. Consistent with the presence of functional homomeric ASIC1a channels, which are Ca2+-permeable, perfusion of acidic solution in these neurons increased intracellular Ca2+ concentration, even in the presence of blockers of voltage-gated Ca2+ channels and glutamate receptors. As expected, the acid-induced increase of intracellular Ca2+ was inhibited by PcTX1 and completely eliminated in neurons from ASIC1 knockout mice. Thus, acidosis can cause intracellular Ca2+ accumulation through activation of homomeric ASIC1a channels, though a secondary activation of other channels cannot be excluded.

To provide a link between ASIC1a activation and ischemic brain injury, both in vitro neuronal injury and in vivo cerebral ischemia models were employed. A brief (1 h) acid incubation, in the presence of blockers of glutamate receptors and voltage-gated Ca2+ channels, was able to induce substantial neuronal injury measured at 6 h or 24 h after acid treatment. This acid-induced, glutamate-independent neuronal injury was inhibited by amiloride or PcTX1, supporting the involvement of homomeric ASIC1a channels. Consistent with an essential role for ASIC1a subunit in acid injury, neurons cultured from ASIC1 knockout mice were resistant to acid incubation. Reducing the concentration of extracellular Ca2+, which decreases the driving force for Ca2+ entry, also ameliorated acid injury. To know whether ASIC1a-mediated injury can also take place in ischemic condition, acid-mediated neuronal injury was studied in the condition of oxygen glucose deprivation (OGD). OGD, in the presence of blockers of glutamate receptors and voltage-gated Ca2+ channels, enhanced the acid-induced neuronal injury which was inhibited by amiloride and PcTX1. Thus, in vitro studies support a role for ASIC1a activation in acidosis-mediated, Ca2+-dependent, ischemic neuronal injury. Since a recent study showed that PcTX1 also inhibits heteromeric ASIC1a/ASIC2b channels in addition to homomeric ASIC1a channels, the potential contribution of heteromeric ASIC1a/ASIC2b channels to acidosis-mediated neuronal injury cannot be excluded.

Does activation of ASIC1a channels also play a role in ischemic brain injury in vivo? To answer this question, two sets of experiments were performed. The first experiment examined whether application of ASIC inhibitors reduces ischemic brain injury, and the second tested whether knockout of the ASIC1 gene renders the animal resistant to cerebral ischemia. Rodent model of focal ischemia, by middle cerebral artery occlusion (MCAO), was employed for both experiments. In rats and mice, intracerebroventricular injection of ASIC1a inhibitor PcTX1 reduced the infarct volume by up to 60%, measured at 24 h after MCAO. Similar to the pharmacological blockade, ASIC1 gene knockout provided a comparable degree of protection against ischemic brain injury. Remarkably, in contrast to most glutamate antagonists which have only a short time window of <1 h, the protection by ASIC1a blockade showed a prolonged effective time window of ~5 h.

In addition to rodent cells, functional Ca2+-permeable ASICs have been recently described in human brain neurons. Activation of these channels, as expected, contributed to acidosis-mediated injury.
of human brain neurons in culture. Thus, Ca\(^{2+}\)-permeable ASIC1a channels represent a promising therapeutic target for human cerebral ischemia.

**ISCHEMIA-RELATED SIGNALS ENHANCE THE ACTIVATION OF ASICs**

Even though the results from both *in vitro* and *in vivo* pharmacological and molecular biological interventions clearly supported an important role for ASIC1a activation in neuronal injury associated with cerebral ischemia, several questions remained to be addressed.

Under *in vitro* experimental conditions, currents of most ASIC subtypes, particularly the homomeric ASIC1a channels, decay rapidly in the continuous presence of acidic pH,\(^{23}\) a phenomena of channel desensitization. In addition, pre-exposure of these channels to small pH drops (e.g., from 7.4 to 7.2) that are insufficient to activate the channel also suppresses the channel activity in response to subsequent, large pH drops, a process termed steady-state desensitization.\(^{24,25}\) Furthermore, it has been shown that the activities and/or expression of some ion channels could be dramatically down-regulated following the ischemia/hypoxia, as exemplified by N-methyl d-aspartate (NMDA) channels in hypoxic turtle brain.\(^{26}\) Thus, whether a significant amount of ASIC1a current can be activated in ischemic conditions and whether the effects of ASIC1a activation (e.g., membrane depolarization and intracellular Ca\(^{2+}\) accumulation) could be long-lasting and detrimental are crucial in determining the pathological functions of these channels.

To this end, Xiong and colleagues first showed that OGD treatment can dramatically potentiate the activity of neuronal ASICs—the amplitude of the acid-activated current was enhanced while the decay of the current was reduced following 1 h OGD.\(^{15}\) The overall outcome of these two effects would be a dramatically increased ASIC-mediated response, which might be translated into enlarged and longer-lasting intracellular Ca\(^{2+}\) elevation. What could be the underlying mechanisms responsible for the changes of electrophysiological property of ASICs observed after OGD or ischemia? This question was not directly answered by the studies of Xiong et al. However, later studies by different laboratories have provided important missing links. It is now known that various ischemia-related signals, for example, arachidonic acid, dynorphine, lactate, and spermine, can dramatically enhance the amplitude and/or reduce the desensitization of ASICs\(^{27}\) (Figure 1).

**Arachidonic Acid**

Arachidonic acid (AA) is a polyunsaturated fatty acid present in the phospholipids of all cell membranes and one of the most abundant fatty acids in the brain. In

![Figure 1](image-url)
addition to being involved in cellular signaling as a lipid second messenger, AA plays important roles in pathological conditions including brain ischemia. Following brain ischemia, the rise of [Ca$^{2+}$], leads to activation of phospholipase A2, resulting in increased production of lipid mediators including AA. Although the exact mechanisms are unclear, high concentrations of lipid mediators are known to cause neurotoxicity.

On the basis of demonstrated effects of AA on a variety of voltage-gated and ligand-gated ion channels, for example, potentiation of NMDA channel currents, Allen and Attwell tested the effect of AA on ASICs. In rat cerebellar Purkinje cells, bath perfusion of 5 or 10 μM AA produced a large increase in the amplitude of the ASIC current. In addition to potentiating the peak amplitude, AA enhanced or induced an additional sustained component of the current. In heterologous expression systems, AA potentiates both homomeric ASIC1a and ASIC2a channels. Thus, promoting the activation of ASIC1a channels could be one of the mechanisms mediating the neurotoxicity of AA.

CaMKII phosphorylation of ASIC1a with KN-93, or mutation of ASIC1a at Ser478 and Ser479, produced neuroprotection. Thus, phosphorylation of ASIC1a by CaMKII deteriorates ASIC-mediated neuronal injury in ischemia.

**Dynorphins**

Dynorphins are endogenous neuropeptides abundantly expressed in the central nervous system (CNS). They are involved in a variety of physiologic functions. Under pathophysiological conditions where their levels are substantially elevated, these peptides can be neurotoxic, partially mediated through glutamate receptors. Recently, Sherwood and Askwith reported that, at high concentrations dynorphins such as big dynorphine potentiate acid-activated currents in mouse cortical neurons and in CHO cells expressing homomeric ASIC1a channels. The potentiation of the ASIC1a activity was mediated through a reduction of the steady-state desensitization of these channels. In the absence of big dynorphine, pre-exposing neurons to conditioning pH of 7.0 completely desensitizes the channels, resulting in no responses to subsequent larger decrease in pH (e.g., to 5.0). In the presence of big dynorphine, however, ASIC1a currents were readily activated. As expected, big dynorphine enhanced ASIC1a-mediated neuronal injury during prolonged acidosis.

**Lactate**

Back in 2001, Immke and McCleskey demonstrated that, in sensory neurons that innervate the heart and COS-7 cells transfected with AIC1a channels, addition of lactate, at the level seen in ischemia, dramatically increased the amplitude of the ASIC current activated by a moderate pH drop to ~7.0. The increase of the current amplitude was accompanied by a reduced current desensitization. Applications of lactate at pH values that do not activate ASICs caused no response. Thus, lactate acted by potentiating but not activating the ASICs. The effect of lactate persisted in excised membrane patches indicating the lack of second messenger involvement. As lactate has the ability to chelate the divalent cations, which have a modulatory role for various membrane receptors and ion channels, it was logical to hypothesize that potentiation of the ASIC currents could be due to a chelation of Ca$^{2+}$ and Mg$^{2+}$ in the solution. Indeed, adjusting the concentrations of Ca$^{2+}$ and Mg$^{2+}$ eliminated the potentiating effect of lactate. Similar to the cardiac sensory neurons, potentiation of the ASIC current by lactate has been reported in other neurons.
**TABLE 1 | Ischemia-related Endogenous Modulators Known to Potentiate ASIC-mediated Responses**

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Neurons or ASIC subunit tested effective</th>
<th>Modulation site(s)</th>
<th>Functional outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>Cerebellar Purkinje cells Sensory neuron ASIC1a ASIC3</td>
<td>Unknown</td>
<td>Increased amplitude Reduced desensitization</td>
<td>32, 60</td>
</tr>
<tr>
<td>CaMK II</td>
<td>Hippocampal neurons ASIC1a</td>
<td>Intracellular S478 and S479</td>
<td>Increased amplitude Increased injury</td>
<td>34</td>
</tr>
<tr>
<td>Dynorphine</td>
<td>Cortical neurons Hippocampal neurons ASIC1a ASIC1b</td>
<td>Extracellular PctX1 site</td>
<td>Reduced steady-state desensitization Increased acid-injury</td>
<td>36</td>
</tr>
<tr>
<td>Lactate</td>
<td>Sensory neuron, Cerebellar Purkinje neurons ASIC1a ASIC3</td>
<td>Extracellular</td>
<td>Increased amplitude Reduced desensitization</td>
<td>37, 60</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>DRG neurons Neuro2A cells ASIC1a ASIC1b ASIC2a ASIC3</td>
<td>Extracellular</td>
<td>Increased current amplitude Increased acid-injury</td>
<td>45, 61</td>
</tr>
<tr>
<td>Protease</td>
<td>Hippocampal neurons ASIC1a</td>
<td>Extracellular PctX1 site</td>
<td>Enhanced current amplitude activated from a baseline pH of 7.0 Increased recovery from desensitization</td>
<td>48</td>
</tr>
<tr>
<td>Spermine</td>
<td>Cortical neurons Hippocampal neurons ASIC1a</td>
<td>Extracellular PctX1 site E219 and E242</td>
<td>Increased amplitude Reduced desensitization Reduced steady-state desensitization Increased recovery from desensitization Increased acid-injury</td>
<td>56, 57</td>
</tr>
</tbody>
</table>

**Nitric oxide**

Nitric oxide (NO) is an important reactive oxygen/nitrogen species which has a variety of physiological and pathological functions. During ischemia, intracellular Ca$^{2+}$ overload leads to activation of the Ca$^{2+}$-dependent neuronal form of nitric oxide synthase (nNOS), resulting in an increased production of NO. NO can also be released by activated microglia. Excessive NO production is known to increase neuronal injury. Although the formation of a strong oxidant of peroxynitrite is likely involved in cell injury, other mechanisms cannot be excluded. Cadiou and colleagues reported that NO donor S-nitroso-N-acetylpenicillamine (SNAP) potentiates ASIC currents in DRG neurons and in CHO cells expressing ASIC subunits. Modulators of the cGMP/PKG pathway had no effect on the potentiation, but in excised patches from CHO cells expressing ASIC2a, the potentiation could be reversed by externally applying reducing agents. NO, therefore, has a direct external effect on ASICs, probably through oxidization of cysteine residues.

**Proteases**

Brain ischemia is accompanied by increased protease activity. Following ischemia, blood-derived proteases have access to the interstitial space from a compromised blood–brain barrier. Studies by Poirot and colleagues demonstrated an ASIC1a-specific modulation of the ASIC activity by serine proteases. Exposure of cells to trypsin, for example, leads to a decreased ASIC1a current if the channel is activated by a pH drop from pH 7.4. However, if acidification occurs from a lower basal pH (e.g., 7.0), a condition pertinent to brain ischemia, protease exposure increases, rather than decreases, the ASIC1a activity. Further studies demonstrate that trypsin modulates the ASIC1a function by cleaving this subunit at Arg-145, which is in the N-terminus of the extracellular loop overlapping with the PctX1 binding site.
Spermine

Spermine is a polyvalent cation involved in various physiological processes. High concentration of spermine can induce neuronal depolarization and cytoplasmic Ca\(^{2+}\) overload, which may lead to neuronal damage.\(^{50}\) Following ischemia, the activity of ornithine decarboxylase (ODC), a rate-limiting enzyme responsible for polyamine synthesis is enhanced, leading to elevated level of spermine.\(^{51}\) Although the modulation of NMDA receptor function\(^{52,53}\) might explain its neurotoxicity, several studies have yielded inconsistent results.\(^{54,55}\)

Babini and colleagues demonstrated that spermine potentiates the activities of ASICs.\(^{56}\) More recently, Duan and colleagues showed that extracellular spermine exacerbated ischemic neuronal injury through sensitization of ASIC1a channels to acidosis.\(^{57}\) In addition to increasing channel activation, spermine reduced channel desensitization and accelerated recovery from desensitization in response to repeated acid stimulation. Thus, extracellular spermine contributes to ischemic neuronal injury, at least in part, by enhancing the activity of ASIC1a channels.\(^{57}\)

CONCLUSION

Stroke or cerebral ischemia is a leading health problem worldwide. Unfortunately, there is still no effective treatment for stroke patients. Searching for new brain injury mechanisms and effective therapeutic strategies is a major challenge and priority. Acidosis is a primary feature associated with cerebral ischemia and is known to cause brain injury. The finding that activation of ASICs contributes to acidosis- and ischemia-induced intracellular Ca\(^{2+}\) accumulation and neuronal injury has suggested that these channels may represent potential new therapeutic targets for stroke intervention. In additional to developing pharmacological agents directly targeting ASICs, alternative strategies can be considered by targeting ischemia-related signals known to potentiate the activity of these channels (Table 1). Alternative neuroprotective strategies may also consider targeting the mechanisms and pathways that control the expression level of total protein and/or surface ASIC1a.\(^{58,59}\)

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