Extracellular Zinc Protects Against Acidosis-Induced Injury of Cells Expressing Ca\textsuperscript{2+}-Permeable Acid-Sensing Ion Channels

Jessica G. Hey, BS; Xiang-Ping Chu, MD, PhD; Joshua Seeds, MSc; Roger P. Simon, MD; Zhi-Gang Xiong, MD, PhD

Abstract—Acidosis is a common feature of neurological conditions including brain ischemia, epileptic seizures, and neurotrauma. Activation of Ca\textsuperscript{2+}-permeable acid-sensing ion channels (ASIC1a) is involved in acidosis-mediated ischemic brain injury. Zn\textsuperscript{2+} is a divalent cation concentrated in nerve terminals in various brain regions, and is released into the extracellular space during excitatory stimulation. Our previous studies have demonstrated that the activities of ASIC1a containing channels and acid-induced increased intracellular Ca\textsuperscript{2+} concentrations are inhibited dramatically by the physiological concentration of extracellular Zn\textsuperscript{2+}. In this report, we demonstrate that decreasing the concentration of the extracellular Zn\textsuperscript{2+} significantly enhances acid-induced injury of HEK 293 cells, a cell line expressing homomeric ASIC1a-like channels, whereas increasing the concentration of extracellular Zn\textsuperscript{2+} appears to be protective. Although increased concentrations of intracellular Zn\textsuperscript{2+} have been shown to be detrimental to neurons, our findings may suggest that the physiological concentration of extracellular Zn\textsuperscript{2+} might play a protective role in acidosis-induced, ASIC1a-mediated neuronal injury. (Stroke. 2007;38[part 2]:670-673.)

Key Words: acid ■ cell injury ■ HEK293 ■ zinc

Acidosis, or significant decrease of tissue pH, is common in brain in acute neurological disorders, particularly in stroke.\textsuperscript{1,2} For several decades, acidosis has been known to be associated with neuronal injury.\textsuperscript{1} However, the detailed mechanism underlying acid-mediated neuronal injury remained elusive. Previously, we demonstrated that activation of Ca\textsuperscript{2+}-permeable acid-sensing ion channels (ASIC1a) and subsequent intracellular Ca\textsuperscript{2+} accumulation is largely responsible for acidosis-mediated neuronal injury,\textsuperscript{3,4} disclosing a novel potential therapeutic target for stroke intervention.\textsuperscript{5,6} In addition, we have shown that the activities of ASICs are modulated by endogenous signaling molecules.\textsuperscript{7,8} For example, the activities of ASIC1a containing channels are substantially inhibited by physiological concentrations of Zn\textsuperscript{2+}, a divalent cation concentrated in excitatory nerve terminals and released into the extracellular space during stimulation.\textsuperscript{7,9} Because activation of ASIC1a channels is involved in acidosis-mediated neuronal injury, this finding suggests that extracellular Zn\textsuperscript{2+} may reduce acidosis-mediated injury of cell expressing ASIC1a channels. Because native neurons in the brain contain not only homomeric ASIC1a but also heteromeric ASIC1a/ASIC2a and homomeric ASIC2a channels,\textsuperscript{3,10} and the fact that Zn\textsuperscript{2+} has dramatically different effect on different configurations of ASICs,\textsuperscript{7,11} we decide to use HEK 293 cells, a cell line containing only ASIC1a-like channels\textsuperscript{12} to test the effect of Zn\textsuperscript{2+} on acid-induced injury.

Methods

Cell Culture

HEK 293 cells were plated in 24-well culture plates and 35-mm culture dishes with minimal essential medium supplemented with 10% fetal bovine serum. Cells were used for electrophysiological recording and cell injury assays 3 to 5 days after plating.

Electrophysiology

Whole-cell voltage-clamp recordings were performed using Axopatch-1D amplifier (Molecular Device). Extracellular solution contained (in mM): 140 NaCl, 5 KCl, 20 HEPES, 10 glucose, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}. The pH was adjusted with NaOH or HCl. Osmolarity was adjusted to 320 to 330 mOsm. Patch electrodes contained (in mM): 140 CsF, 2.0 MgCl\textsubscript{2}, 1.0 CaCl\textsubscript{2}, 10 HEPES, 11 EGTA, 4 MgATP, with pH adjusted to 7.3 and osmolarity to ~300 mOsm.

Cell Injury Assay

Lactate dehydrogenase (LDH) release was measured using cytotoxicity detection kit (Roche). Optic density was measured at 490 nm with subtraction of the reference value at 620 nm. Propidium iodide and fluorescein diacetate were used for the staining of alive and dead cells, respectively, and were detected by a fluorescent microscope with excitation/emission wavelength at 530/612 nm for propidium iodide and 500/550 nm for fluorescein diacetate.

Results

Extracellular Acidosis Activates ASIC1a-Like Channels in HEK293 Cells

We first studied the existence of ASIC current in HEK293 cells using patch-clamp recording and fast-perfusion tech-
Decreasing the extracellular pH (pHₐ) from 7.4 to 6.0 activates transient inward current in all cells recorded (Figure 1), as demonstrated previously.¹² The current is sensitive to the blockade by amiloride, a nonspecific ASIC blocker (not shown), indicating activation of ASICs in these cells. The acid-activated current in HEK293 cells is largely blocked by PcTX venom (150 ng/mL), a specific blocker for homomeric ASIC1a channels,¹ suggesting that the acid-activated current in these cells is mediated by homomeric ASIC1a channels (Figure 1).

Zinc Chelator Potentiates Acid-Activated Current in HEK293 Cells

We then determined whether changing the concentration of the extracellular Zn²⁺ affects the amplitude of ASIC current in HEK293 cells. Bath application of TPEN, a high-affinity Zn²⁺ chelator,¹³ dramatically potentiated the ASIC current by ≈50% (n=5, Figure 1). In contrast, addition of 10 μmol/L ZnCl₂ slightly inhibited the ASIC current (Figure 1). The lack of dramatic inhibition of the current by addition of 10 μmol/L ZnCl₂ is likely attributable to the fact that the ambient concentration of Zn²⁺ present in most physiological solutions (eg., ≈50 nM) is already near the saturating concentration for high affinity Zn²⁺ inhibition of the ASIC1a current.⁷

Acid Injury of HEK293 Cells

Next, we studied acid injury of HEK293 cells. HEK293 cells grown in 24-well plates were randomly divided into different treatment groups and incubated with either normal (pH 7.4) or acidic (pH 5.5) solutions. Cell injury was determined by LDH release measured at different time points following 3 hour of acid incubation. As shown in Figure 2, acid incubation induced a time-dependent increase in LDH release compared with cells treated with normal extracellular solutions, indicating injury of HEK293 cells by acidosis. Incubation of cells with pH 6.0 solution also induced significant increase of LDH release (n=4, not shown).

Acid Injury of HEK293 Cells Is Inhibited by PcTX Venom

We then determined whether acid-induced injury of HEK293 cells can be inhibited by PcTX venom; 150 ng/mL PcTX venom was included in both normal and acidic solutions. Addition of 150 ng/mL PcTX venom slightly increased the baseline LDH release at pH 7.4; however, it significantly reduced the LDH release induced by incubation with acidic solutions (Figure 3), suggesting that acid-induced injury of HEK293 cells is largely caused by activation of homomeric ASIC1a channels.
Acid-Induced Injury of HEK293 Cells Is Potentiated by Zn\(^{2+}\)/H\(_{11001}\) Chelation

We next determined whether changing the concentration of extracellular Zn\(^{2+}\)/H\(_{11001}\) has an effect on acid-induced injury of HEK293 cells. The Zn\(^{2+}\)/H\(_{11001}\) chelator TPEN, at 10 \(\mu\)mol/L, was added to the treatment solutions. Addition of 10 \(\mu\)mol/L TPEN did not significantly affect the baseline LDH release at 7.4; however, it dramatically potentiated the LDH release induced by incubating cells with acidic solutions (Figure 4). In contrast, addition of 10 \(\mu\)mol/L ZnCl\(_2\) slightly increased acid-induced LDH release.

Discussion

Brain acidosis, characterized by an increase in proton concentration or decrease in tissue pH, is an important component of the pathogenic events associated with various neurological conditions including brain ischemia.\(^14\) The decrease of brain pH, even moderately, is expected to activate ASICs, especially the Ca\(^{2+}\)/H\(_{11001}\)-permeable ASIC1a channels that are highly expressed in neurons of the central nervous system. Activation of these channels results in membrane depolarization and intracellular Ca\(^{2+}\) accumulation, which contributes substantially to acidosis-mediated ischemic brain injury.\(^3,4\)

Zn\(^{2+}\), an endogenous divalent cation and trace element, is stored at presynaptic terminals in various brain regions and released during intense neuronal excitation.\(^9,15\) The role of these Zn\(^{2+}\) stores in neuronal injury is not clear. Although abnormal increases of extracellular Zn\(^{2+}\) are associated with neurotoxicity\(^16,17\) likely caused by entry of Zn\(^{2+}\) into neurons,\(^18\) high-affinity inhibition of ASIC1a channels, as shown by our studies,\(^7\) along with Zn\(^{2+}\) inhibition of NMDA channels,\(^19,20\) strongly suggest that moderate increase of the extracellular Zn\(^{2+}\) may serve as a negative feedback mechanism to maintain neuronal excitation at a moderate level thus protecting neurons from excitatory and acidosis-mediated injury.

This notion is consistent with a previous report that disrupting Zn\(^{2+}\) storage in synaptic vesicles, by knock-out of the Zn\(^{2+}\) transporter, rendered mice more susceptible to kainic acid-induced seizures.\(^21\) Similarly, studies by Blasco-Ibanez et al demonstrated that chelation of synaptic zinc induced over-excitations of hilar mossy cells in rat hippocampus.\(^22\)
In summary, our present studies strongly suggest that in pathological conditions in which severe acidosis takes place, a moderate increase of Zn\(^{2+}\) in the extracellular space may serve as a neuroprotective measure against acid injury through inhibition of Ca\(^{2+}\)-permeable ASIC1a channels. It is, however, worth mentioning that the current study using HEK 293 cells is largely for “proof of principle,” which cannot be directly translated to the stroke brain. The HEK cells are generally tolerant to various injury paradigms and the pH 5.5 used in this study is unlikely to be achieved in the ischemic brain. Future studies will need to use primary neurons, or at least neuronal cell lines to confirm these findings.

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Disclosures

None.

References

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