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Inhibition of acid-induced apoptosis by targeting ASIC1a mRNA with short hairpin RNA[1]

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Key words

acid-sensing ion channel; RNA interference; short hairpin RNA; apoptosis

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Abstract

Aim: To study the role of acid-sensing ion channel (ASIC) 1a in the cell death and apoptosis induced by extracellular acid in C6 glioma cells. **Methods:** The stable ASIC1a-silenced C6 cell line, built with RNA interference technology, were confirmed by RT–PCR and Western blot analysis. The cell viability following acid exposure was analyzed with lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The apoptotic cells dyed with Annexin-V and propidium iodide were measured with a flow cytometer, while the changes of cell cycle were also assayed. **Results:** The downregulation of ASIC1a proteins by stable transfection of short hairpin RNA decreased the cell death percentage and increased cell viability following acid exposure with LDH and the MTT assay. The rate of apoptosis was lower in the ASIC1a-silenced cell line than that in the wild-type C6 cell line. The percentage of sub-G_0 cells was lower in the ASIC1a-silenced C6 cells than that in the wild-type cells. **Conclusion:** Extracellular acid induced cell death and apoptosis via ASIC1a mechanisms in the C6 glioma cells.

Introduction

Acid-sensing ionic channels (ASIC) are H^+-gated cation channels expressed throughout mammalian central and peripheral nervous systems[1–6]. They belong to the degenerin/epithelial sodium channel superfamily. Until now, 6 ASIC subunits have been cloned: ASIC1a[1], ASIC1b[2], ASIC2a[3], ASIC2b[7], ASIC3[1,2,8], and ASIC4[9]. Functional ASIC are usually tetrameric assemblies of these ASIC subunits in homomeric or heteromeric conformation[10]. However, neither ASIC2b nor ASIC4 can form functional homomeric channels themselves[7,9,11]. ASIC2b has been shown to associate with other subunits and modulate their activity[7]. Various ASIC exhibit different kinetics, pH sensitivities, and tissue distribution[7,10,12]. The pH of half-maximal activation ($pH_{0.5}$) of these channels differs: ASIC1a (or ASIC1), $pH_{0.5}$=6.2[1]; ASIC1b, a splice variant of ASIC1a, $pH_{0.5}$=5.9[13]; ASIC2a, $pH_{0.5}$=4.4[10]; and ASIC3, $pH_{0.5}$=6.5[2]. Besides Na^+ permeability, homomeric ASIC1a can flux Ca^{2+}[1,14,15].

Both ASIC1a and ASIC2a subunits have been shown to be abundant in the brain, whereas ASIC2b is present in both brain and sensory neurons[5,16–18]. ASIC4 is found to be co-expressed with other subunits in many areas of the brain. ASIC3 is expressed almost exclusively in sensory neurons in rats and generates a biphasic current with a fast, inactivating phase, followed by a sustained component[2,4,12].

Under most pathological conditions ranging from ischemia, inflammation, epilepsy to cancer, tissue pH often falls to about pH 6.0[19-21]. Acidosis is assumed to play a critical role in cell injury[21]. Although the detailed mechanisms remain unclear, ASIC are thought to be involved in this process. In sensory neurons, ASIC have been proven to play an important role in nociception during a tissue acidosis, for instance in muscle and cardiac ischemia[22] and in inflammation[23]. In peripheral sensory neurons, ASIC have been implicated in mechanosensation[24] and perception of pain during tissue acidosis[25-29], particularly in ischemic myocardium where ASIC are likely transducers of anginal pain[30]. The presence of ASIC in the brain, which lacks nociceptors, suggests that these channels have functions beyond nociception. For example, our previous studies have identified the characteristic of ASIC in the hippocampal

neurons[31]. Now, increasing evidence has proved that ASIC1a is involved in synaptic plasticity, learning/memory, and fear conditioning[18,32] and participates in neuronal damage associated with tissue acidosis[15,33].

Now, ASIC have been found in malignant gliomas[34]. Recent studies have revealed that the surface expression of ASIC2 could inhibit the amiloride-sensitive current and migration of glioma cells[34]. However, the role of ASIC1a in acid-induced cytotoxicity remains undefined. Here, using the specific technology of RNAi (RNA interference), *in vitro* cell toxicity assays, and an apoptosis study, we found that extracellular acid induced cell death and apoptosis in the rat C6 glioma cell line. Data also showed that reducing ASIC1a proteins by stable expression of short hairpin RNA (shRNA) significantly decreased acid-induced cytotoxicity.

Materials and methods

Chemicals and antibodies Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co (St Louis, MO, USA). The antibody against ASIC1a (category No ASIC11-A) was purchased from Alpha Diagnostics International (San Antonio, TX, USA).

Cell culture The rat glioma C6 cell line derived from rat neurogliocytoma was purchased from the Cell Culture Centre of the Institute of Basic Medical Sciences (Chinese Academy of Medical Sciences, Beijing, China). The C6 cell line was cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.03% *L*-glutamine (*w*/*v*), and 2.2% sodium bicarbonate (*w*/*v*). The ASIC1a-silenced cells and the control cells were screened with the G418. The cells were cultured at 37 °C a in a humidified atmosphere with 5% CO_2 (310/Thermo, Forma Scientific, Marietta, OH, USA). G418, penicillin, *L*-glutamine, nimodipine, (+/–)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d] cyclohepten-5,10-imine (MK801), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and all other reagents were purchased from Sigma (St Louis, MO, USA).

Analysis of cell death The cells were plated in 96-well plates at a density of 1×10^4 cells/well and incubated for 16–20 h. Then the cells were treated with acid incubation for 3 h in RPMI-1640 medium with 10 μmol/L MK801, 20 μmol/L CNQX, and 5 μmol/L nimodipine, which were added to eliminate the effect of glutamate receptors and voltage-gated Ca^{2+} channels. After drug treatment, the cells were washed twice with phosphate buffered saline (PBS) and were recultured in complete RPMI-1640 medium containing 10% FBS for about 4 h. Subsequently, the medium was replaced and the cells were incubated with 0.5 mg/mL MTT in complete RPMI-1640 medium for 4 h[15]. The surviving cells converted MTT to formazan, which generates a blue–purple color when dissolved in DMSO. The intensity was measured at 490 nm using a plate reader (Molecular Dynamics Inc, Sunnyvale, CA, USA) for the enzyme-linked immunosorbent assays. The relative percentage of survival was calculated by dividing the absorbance of the treated cells by that of the control in each experiment.

Cell injury assay

Lactate dehydrogenase (LDH) release assay The degree of cell injury was assessed by a quantitative measurement of released lactate dehydrogenase (LDH). The cells were treated with acid incubation as described earlier. Then the culture medium was removed and the cells were incubated with necessary cofactors as described by the manufacturer's assay kit (Sigma Diagnostics, St Louis, MO, USA). One percent of Triton X-100 in PBS was used to extract the remaining LDH from the cultures. Absorbance measurements at 440 nm were taken every 30 s for 3 min by using a 96-well microplate reader (Molecular Dynamics, USA). The resulting slopes defined the LDH units per sample. Data were expressed as a ratio of LDH released/(LDH released+LDH Triton-X extracted).

Apoptosis assay After exposure to the extracellular solution (pH 6.0) for 3 h, the cells continue to be cultured in the extracellular solutions (pH 7.4) for about 4 h[15]. In order to eliminate the effect of glutamate receptors and voltage-gated Ca^{2+} channels, MK801 (10 μmol/L), CNQX (20 μmol/L), and nimodipine (5 μmol/L) were also added to these solutions. Then the cells were washed with cold PBS and resuspended with binding buffer [10 mmol/L hydroxyethyl piperazine ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$]. The solution containing 10^5 cells was transferred to a 5 mL tube, and 5 μL of Annexin-V–fluorescein isothiocyanate (FITC) and 10 μL PI were added to the culture medium and incubated for 30 min in the dark. Binding buffer was then added to each tube and the apoptotic cells were assessed with the flow cytometer.

Cell cycle analysis The cells were cultured and treated as indicated earlier. Then the cells were harvested, washed with PBS, and fixed in 70 % ethanol. After being fixed for 12 h at –20 °C, the cells were collected by centrifugation and resuspended in 1 mL staining solution containing 50 μg/mL PI, 1 mg/mL RNase A, and 1 mg/mL glucose in PBS. The cells were analyzed for DNA content using a XL flow cytometer (Beckman Coulter Inc, Miami, FL, USA) after

being incubated for 1 h of staining.

Western blot analysis Western blot analyses of ASIC1a were performed using the antibody against ASIC1a (category No ASIC11-A). A cell lysate containing 30 μg protein was fractionated by SDS–PAGE and then transferred to a nitrocellulose membrane. The membrane was first rinsed with TBST [20 mmol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, and 0.05% Tween 20] and then blocked with 5% (*w*/*v*) skim milk in TBST for 1 h at room temperature. The blocked membrane was subsequently probed for 1 h at room temperature with the first antibody at a dilution of 1:1000 in the blocking buffer. Then the membrane was washed 3 times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated antibodies against rabbit immunoglobulin G (IgG). After the membrane had been washed with TBST, bands of protein on the membrane were visualized using the enhanced chemiluminescence detection system (PerkinElmer Life Sciences, Boston, MA, USA).

RT–PCR Total RNA was purified with RNeasy columns (Qiagen, Valencia, CA, USA). One-step RT–PCR was performed in 50 μL reactions using 100 ng RNA with 0.5 μmol/L of each primer. The annealing temperature was set at 53 °C and repeated for 25 cycles. Other PCR conditions and reagents were supplied and recommended by the manufacturer's protocol for the Titan one-step system (Roche Applied Science, Indianapolis, IN, USA). The primer sequences[2,4,5,8,9,11,12] for ASIC1a were as follows: 5'-CGGATCCATGGAATTGAAGACCGAGGA-3' (forward) and 5'-CGATATCTGCAGGTAAAGTCCTCAAACG-3' (reverse); ASIC2a: 5'-CGAATTCATGGACCTCAAGGAAAGCC-3' (forward)and5'-GCTCGAGGCAGGCAATCTCCTCCAGGG-3' (reverse); ASIC1b: 5'-CAGGGTCGGAGTGGATGAG-3' (forward) and 5'-GAGCGATTATAGAAACGATGGAG-3' (reverse); ASIC2b: 5'-CGAATTCATGAGCCGGAGCG-GCGGAGC-3'(forward)and5'-GCTCGAGGCAGGCAATCTC-CTCCAGGG-3' (reverse); ASIC3: 5'-CGCGAATTCATGA-AACCTCGCTCCGGACTG-3' (forward) and 5'-GCGCTC-GAGGAGCCTTGTGACGAGGTAAC-3'(reverse);ASIC4:5'-CGCGAATTCATGCCGATCGAGATTGTGTGC-3'(forward) and5'-GCGCTCGAGGCAAGCAAAGTTTTCAAAGAG-3' (reverse);andGAPDH:5'-GTCAACGGATTTGGTCGTATTG-3' (forward) and 5'-AGTGATGGCATGGACTGTGGT-3' (reverse).

shRNA design and preparation The design of small interference RNA (siRNA) was based on Dharmacon siDESIGN Center software (Dharmacon., Lafayette, CO, USA). The sequence for ASIC1a RNAi was 5'-GATCCCGCGTGAA-TTCTACGACAGATTCAAGAGATCTGTCGTAGAA-TTCACGCTTTTT-3' (forward) and 5'-AGCTAAAAAG-CGTGAATTCTACGACAGATCTCTTGAATCTGTCGTAG-AATTCACGCGG-3' (reverse) and the sequence for the control RNAi was 5'-GATCCCTTCTCCGAACGTGTCA-CGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTT-3' (forward) and 5'-AGCTAAAAATTCTCCGAACGTG-TCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGG-3' (reverse). The shRNA were synthesized in desalted and purified form by Dharmacon Research (Lafayette, CO, USA).

Construction of plasmid vectors and transfection The synthesized shRNA fragment containing the open reading frame of interfering-ASIC1a expression cDNA was inserted into the *Hind*III site of the pGCsi–U6–Neo–GFP cloning vector. The insert was digested with *Bam*H I and *Hind*III and subcloned into the *Bam*HI–*Hind*III restriction sites of the pGCsi expression vector. The C6 cells were transfected with the expression plasmids using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions. After transfection, the cells were subjected to the generation of the stable shRNA-transfected C6 cell line.

Generation of stable shRNA-transfected C6 cell line The rat shRNA-transfected plasmid for silencing the ASIC1a gene was earlier described. After transfection, the individual G418-resistant clones were selected. Under the G418 (800 μg /mL) selection, the cells were screened for 20 generations and confirmed to build the stable ASIC1a-knockdown C6 cell line with Western-blotting. After selection with G418, a stably transfected cell line of the ASIC-knockdown C6 cell line was obtained.

Statistical analysis Data were presented as mean ± SEM. Values were compared using either Student's *t*-test or ANOVA. Two-way ANOVA was used to separate the effects of 2 variables and determine their interaction. $P<0.05$ was considered statistically significant.

Results

Successful build of the ASIC1a-silenced C6 cell line In the rat C6 glioma cell line, the transcription level of ASIC1a, ASIC1b, ASIC2a, and ASIC2b was identified with RT–PCR. The transcription of ASIC3 and ASIC4 was not detected in the cell line (Figure 1). To elucidate the role of ASIC1a in the acidosis, a U6 promoter-driven RNAi was used to silence the transcription of the ASIC1a gene in the C6 glioma cells. We designed and prepared the shRNA sequences directly against ASIC1a, and the control sequence was designed according to the Dharmacon siDESIGN Center software. Then we constructed the plasmid vectors with the pGCsi–U6–Neo–GFP cloning vector. The C6 cells were cotransfected with pGCsi–U6–Neo–GFP and either an ASIC1a-

2specific RNAi or a control RNAi expression vector. Stably-transfected clones were selected with G418 and were screened for ASIC1a protein knockdown (C6–sh1-4a). Western blotting showed that a dramatic decrease in ASIC1a protein expression occurred in 1 clone from ASIC1a-specific RNAi (C6–sh1a) compared with those transfected with the vector only (C6–Neo) or the control RNAi clones (C6-negative) (Figure 2).

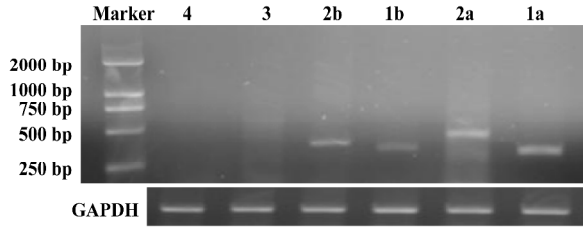

Figure 1. Transcription of different subunits of ASIC in the wild-type C6 glioma cell line by RT–PCR. Total RNA was extracted from the cultured C6 cell line. With specific primers of the 6 ASIC subunits, the RT–PCR analysis was performed according to experiment procedure. PCR products were stained with ethidium bromide (EB) and run on 1% agarose gel. GAPDH (500 bp) was as set as the control.

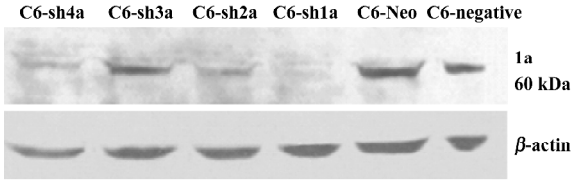

Figure 2. Expression of ASIC1a in different cell lines by Western blot analysis. C6 cells were transfected with either ASIC1a shRNA-expressing plasmid or the pGCsi–U6–Neo–GFP vector alone, and stable clones were selected with G418. Western blotting showed that 1 clone of the ASIC1a-specific RNAi transfection (C6–sh1a) had a dramatic decrease in the ASIC1a protein expression compared with a clone that contained pGCsi–U6–Neo–GFP alone (C6–Neo) or the control clones (C6-negative) that contained a mutated RNAi.

Knockdown of ASIC1a attenuated cytotoxicity induced by extracellular acid In the present study, we tested whether ASIC1a was involved in the neuroglioma cell toxicity when treated with different pH acidic solution. We applied acidic solutions with pH 6.0, pH 6.5, pH 6.9, and pH 7.4 to the C6–sh1a cells and the C6-negative cells, respectively. Cell survival was measured with the MTT assay, while cell injury was tested by LDH release. The experiments were conducted following the earlier-mentioned experiment procedure. To compare the relative percentage of survival conveniently, we took the absorbance of the C6–sh1a cells at pH7.4 as the control. The percentage of cell survival was calculated by dividing the absorbance of the treated cells by that of the control in each experiment. The extracellular acid solution induced cell death in both cell lines. As the pH value decreased, the percentage of survival for the two cell types decreased subsequently (two-way ANOVA, $P<0.05$), which demonstrated that acid stimulus played an important role in cytotoxicity. However, the percentage of survival had obvious distinction between different cell types (two-way ANOVA, $P<0.05$). It indicated that ASIC1a was involved in the cytotoxicity caused by the acid (Figure 3A).

To assay the effect of pH on cell injury, the cells were treated as described earlier. After acid stimulus, the LDH release was measured 24 h later. It was found that exposure to acidic solutions (pH<7.4) induced LDH release in both types of cells compared with exposure to pH 7.4 solution (two-way ANOVA, $P<0.05$; Figure 3B). However, the LDH release in the C6–sh1a cells was much lower compared with that in the C6-negative cells (two-way ANOVA, $P<0.05$; Figure 3B) when treated with incubation in the same acid solution (pH<7.4). It also showed that the cytotoxicity caused by the acid was significantly attenuated by the knockdown of ASIC1a in the C6–sh1a cell line.

Knockdown of ASIC1a decreased the percentage of apoptosis induced by extracellular acid From the above experiments, we found that exposure to acidic solutions increased the cell death of the C6-negative and the C6–sh1a cells. As the percentage of survival of the C6–sh1a cells was much higher than that of the C6-negative cells, it indicated the ASIC1a was involved in the cytotoxicity induced by extracellular acid. We further assessed apoptosis from the cells that had been exposed to extracellular acidic solution. Experiments were conducted according to the earlier-mentioned experiment procedure. Apoptosis was assessed by Annexin-V–FITC staining to detect phosphatidylserine on the cell surface in conjunction with PI staining. Figure 4 shows the result of staining with Annexin-V–FITC and PI on the apoptotic cells and the normal cells (Figure 4). The percentages of apoptosis were very low with the normal pH 7.4 solution (about 1%) and increased with the pH 6.0 solution among the 3 cell types (Figure 4). Further, the C6–sh1a cells showed a decrease in Annexin V-positive and PI-negative cells (6%) when compared with the C6 cells and C6-negative control cells (28% and 24%, respectively). These results indicated the extracellular acid might induce apoptosis by activating ASIC1a.

Cell death induced by acid was associated with the induction of cellular apoptosis To explore the mechanism of low cytotoxicity in ASIC1a-silenced cells exposed to extracellular acid, a cell cycle analysis was also performed. All 3

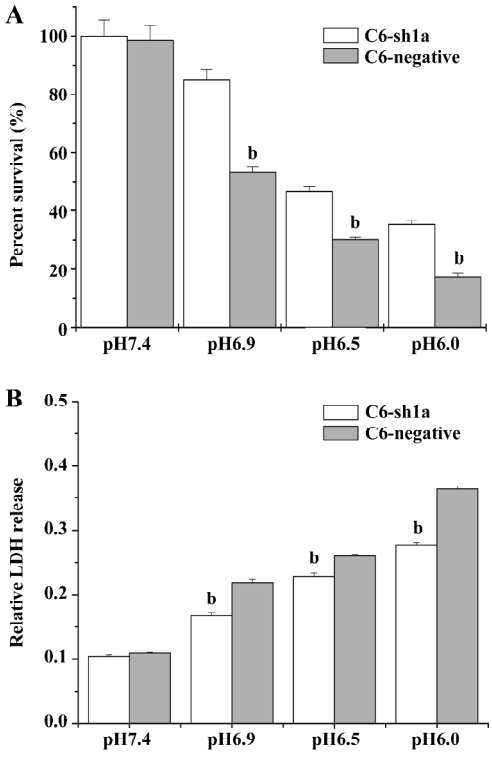

Figure 3. Effects of various pH extracellular solutions on cytotoxicity to both C6-sh1a cells and the C6-negative cells. Both kinds of cells were incubated in different pH extracellular solution. (A) cell survival percentage was measured with MTT assay. Cells were treated with LDH assay, and the survive percentage (%) was assessed 4 h after incubation. Results were obtained from 4–6 experiments, Mean±SEM. $^{b}P<0.05$ *vs* the C6-negative cells (two-way ANOVA). (B) cell injury was assessed by a quantitative measurement of released LDH. Cells were exposed to the acid solution for 3 h, and LDH release was assessed 24 h later. n=12 wells. $^{b}P<0.05$ *vs* the C6-negative cell line at the same pH value (two-way ANOVA).

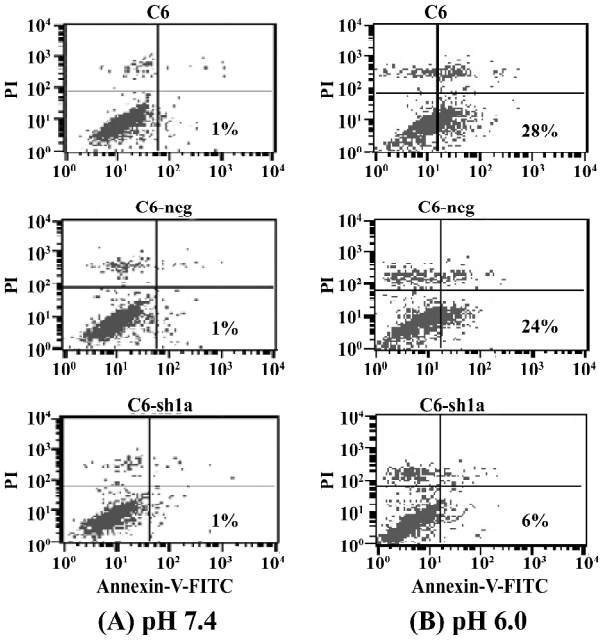

Figure 4. Knockdown of ASIC1a decreased the percentage of apoptosis induced by extracellular acid. After exposure to the extracellular solution of pH 6.0 for 3 h, the cells continued to be cultured in the extracellular solutions of pH 7.4 for about 4 h before the apoptosis analysis. Cells were stained with Annexin-V–FITC and PI to differentiate between apoptosis and necrosis. Viable cells were FITC negative/PI negative, late apoptosis or cell death was FITC positive/PI positive, and early apoptotic cells were FITC positive/PI negative (lower right box with the percentage of population). Profiles showed 1 experiment, which was similar to 2 additional independent experiments. Experiments were conducted according to the experiment procedure mentioned above. (A) wild-type C6 cells, C6-sh1a cells, and the C6-negative cells cultured in the RPMI-1640 medium with normal pH (pH 7.4) were analyzed as the control. (B) wild-type C6 cells, C6–sh1a cells and the C6-negative cells were exposed to the acid RPMI-1640 (pH 6.0). As shown, the percentages of apoptosis of the 3 cell types were low in the normal pH 7.4 solution (about 1%) and increased with the pH 6.0 solution.

cell lines were treated with the same acid solution (pH 6.0) and experiments were conducted following the earlier-mentioned experiment procedure. As shown in Figure 5, the number of cells in the S-phase was 37%, 32%, and 33% for the C6–sh1a, C6, and C6-negative cells, respectively. In normal conditions (pH 7.4), all 3 cell lines had a normal cell cycle distribution with little subdiploid DNA (no more than 1%). Compared with the C6 and C6-negative cells, the C6–sh1a cells showed a remarkable decrease (from 26% to 7%) in the subdiploid DNA (sub-G_0/G_1) content, which suggested that knockdown of ASIC1a decreased cell apoptosis in the acid solution (pH 6.0) (Figure 5). Based on the results from the MTT and LDH experiments, it also indicated that the cell necrosis induced by acid was associated at least in part with the induction of cellular apoptosis.

Discussion

To date, 4 ASIC genes (ASIC1–4) have been identified, among which ASIC1a, ASIC2a, and ASIC2b all have a widespread distribution pattern in the brain. With the use of RT–PCR, we found that ASIC1a, ASIC2a, and ASIC2b were transcribed at high levels in the C6 cell line, which might be the result of their generation from neuroglioma. ASIC3 and ASIC4 were not detected, possibly because of their relatively low expression level in the C6 cell line.

In the present study, we studied the function of ASIC1a with the newly-developed RNAi technique. With the stably ASIC1a-silenced C6 cell line, we found that the extracel-

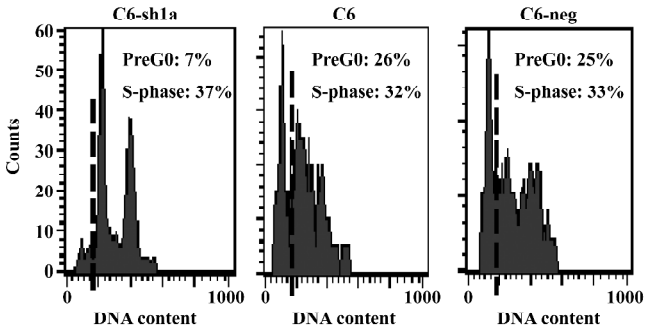

Figure 5. Cell cycle analysis. The 3 cell types were treated with the mentioned experiment procedure before subjected to PI staining and flow cytometry. The figures showed the DNA content of cells after PI staining of methanol-fixed cells. To the right of the dotted line is normal diploid DNA and to the left is sub-G_0/G_1 DNA content (degraded DNA originated from apoptotic cells).

lular acid could induce apoptosis via ASIC1a, which not only suggested that ASIC1a was involved in the apoptosis under the extracellular acid, but also indicated the main reason for cell death.

As we know, with the use of this double-staining regime, it is difficult to distinguish late apoptotic cells from necrotic cells in two-color flow cytometry. In fact, these cells are usually considered as dead cells with flow cytometry or MTT. In our study, the populations of cell death were tested 24 h after the cells were exposed to the sustaining extracellular acid stimulation for 3 or 4 h. However, when we analyzed the 3 populations of cells (non-apoptotic cells, early apoptotic cells, and necrotic cells or late apoptotic cells) only 6 h after exposure to an extracellular acid environment (pH 6.0), nearly one-third of the cells were apoptotic (32%). It was likely that many of the cells would become late apoptotic and dead cells. Thus, it suggested that silencing ASIC1a not only protected the cell from death, but also from apoptosis. It was also likely that ASIC1a was just like a dead receptor in the cellular membrane.

RNAi silences specific gene expression by targeting the relative mRNA and preventing it from being translated into a protein. Our studies were the first to utilize the highly-specific technology of RNAi to directly demonstrate that extracellular acid induced cytotoxicity and apoptosis by activating ASIC1a. As using ASIC1a-deficient mice has been limiting and difficult, the use of ASIC1a shRNA will greatly facilitate the precise identification of the ASIC1a gene function. RNAi mediated by shRNA is a powerful technology that allows the silencing of genes with great specificity and potency. Now it has been used more and more extensively in plants, nematodes, *Drosophila*, and mammalian cells to determine gene function. There are increasing reports on the use of siRNA *in vitro*[36–39] and *in vivo*[40–43]. Although more studies should be done to clarify whether ASIC1a-specific shRNA delivery *in vivo* is biologically effective, our data have shown that the inhibition of ASIC1a expression with RNAi was cytoprotective *in vitro*, which might have significant implications for future therapeutic interventions. As acid-induced cytotoxicity was critical to the pathogenesis of ischemia, inflammation, and tumors in the brain, it was suggested that shRNA for ASIC1a potentially have therapeutic value for which specific, safe antagonists do not currently exist.

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