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The Na^+/HCO_3^- co-transporter is protective during ischemia in astrocytes

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Abstract

The sodium bicarbonate co-transporter (NBC) is the major bicarbonate-dependent acid-base transporter in mammalian astrocytes and has been implicated in ischemic brain injury. A malfunction of astrocytes could have great impact on the outcome of stroke due to their participation in the formation of blood brain barrier, synaptic transmission, and electrolyte balance in the human brain. Nevertheless, the role of NBC in the ischemic astrocyte death has not been well understood. In this work, we obtained skin biopsies from healthy human subjects and had their fibroblasts grown in culture and reprogrammed into human induced pluripotent stem cells (hiPSCs). These hiPSCs were further differentiated into neuroprogenitor cells (NPCs) and then into human astrocytes. These astrocytes express GFAP and S100β and readily propagate calcium waves upon mechanical stimulation. Using pH sensitive dye BCECF [2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein] and qPCR technique, we have confirmed that these astrocytes express functional NBC including electrogenic NBC (NBCe). In addition, astrocytes exposed to an ischemic solution (IS) that mimics the ischemic penumbral environment enhanced both mRNA and protein expression level of NBCe1 in astrocytes. Using IS and a generic NBC blocker S0859, we have studied the involvement of NBC in IS-induced human astrocytes death. Our results show that a 30 μM S0859 induced a 97.5±1.6% (n=10) cell death in IS- treated astrocytes, which is significantly higher than 43.6 ± 4.5%, (n=10) in the control group treated with IS alone. In summary, a NBC blocker exaggerates IS-induced cell death, suggesting that NBC activity is essential for astrocyte survival when exposed to ischemic penumbral environment.

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Keywords

iPSCs; astrocytes; NBC; ischemia; penumbra

Introduction

Ischemic penumbral tissue surrounds the infarct core and is at risk of injury due to the continued presence of ischemic environment (Fisher, 2004). Therefore, understanding the mechanisms of cell death in this region holds great promise for developing strategies for stroke therapy. Multiple cell types are involved in the ischemic penumbra and astrocytes are believed to have great impact on the prognosis of penumbral tissue, especially that astrocytes constitute the most numerous cell type in the mammalian brain. In addition, astrocytes play an important role in maintaining neuronal synaptic transmission, controlling cerebral blood flow, maintaining blood brain barrier integrity and scavenging free radicals, among other roles. Hence, the fate of astrocytes in the ischemic penumbra is critical for the outcome of ischemic stroke.

The Na^+/HCO_3^- co-transporters (NBCs) are bicarbonate-dependent acid base transporters on the cell membrane. NBCs are classified into either electrogenic (NBCe) or electroneutral (NBCn) transporters based on the stoichiometry of HCO_3^- and Na^+ transport (Boron et al., 2009). NBCs are expressed in the mammalian brain with NBCe predominately expressed in the astrocytes (Majumdar and Bevensee, 2010, Theparambil et al., 2014). NBC is involved not only in intracellular pH (pH_i) regulation in astrocytes but also plays a critical role in maintaining astrocytic ionic homeostasis and water balance between the intra- and extra-cellular space in the brain. Under pathological conditions, such as stroke, altered function and/or expression of NBC would have a profound impact on the fate of astrocytes, and therefore brain injury. In a gerbil middle cerebral artery occlusion (MCAO) model, for example, NBCe mRNA and protein expression is increased in the penumbral region and closely related to delayed cell death in the CA1 region of hippocampus. This delayed damage does not occur in CA3 region, where no alteration is detected in NBCe mRNA and protein expression (Sohn et al., 2011). In a permanent MCAO model, the expression of NBCe was found significantly elevated in the penumbra after three hrs of ischemia, suggesting that NBCe could play a role in stroke pathophysiology (Jung et al., 2007).

Although earlier work has related enhanced expression and/or activity of NBC to brain injury, it is unclear as to whether this alteration is causal to injury or whether the alteration is beneficial. For example, previous work has demonstrated that hypoxia/ischemia induces astrocytic injury by changing the expression and function of NBC (Fujita et al., 1999, Khandoudi et al., 2001, Fantinelli et al., 2014). However, others have demonstrated that the alteration in NBC is tantamount to the activation of a protection mechanism for astrocytes (Kumar et al., 2007, Kumar et al., 2011). In this work, we investigate the role of NBC in *human* astrocyte death after exposure to an ischemic solution (IS) that simulates the ischemic penumbral environment (Yao et al., 2007a). We demonstrate that human induced pluripotent stem cells (hiPSCs)-derived astrocytes express a functional NBC and blocking NBC exaggerates ischemic injury in astrocytes. Therefore, our data indicate that the

increased expression of NBC and their activity seen in the ischemic penumbra is a protective mechanism following stroke.

Materials and Methods

Reprogramming of human fibroblast cells and generation of iPSCs and NPCs

Detailed protocols for generating iPSCs and NPCs were described in our previous study (Zhao et al., 2015). In brief, fibroblast cells were generated from skin biopsies of two healthy adult subjects with an informed written consent and under approval by the University of California San Diego. Fibroblast cells were infected with retrovirus vectors containing OCT4, SOX2, KLF4 and c-MYC human cDNAs (Salk Institute Gene Transfer, Targeting and Therapeutics Core, La Jolla, CA). The infected fibroblast cells were then plated onto the irradiated mouse embryonic fibroblast feeder cells incubated with human embryonic stem (ES) cell medium containing 20% knockout serum replacement, 1% non-essential amino acids, 0.2% beta-mercaptoethanol, and 30 ng/ml FGF2. Three weeks later, the iPS colonies were manually picked and maintained in the mTeSR™ medium (StemCell Technologies, Canada). To obtain NPCs, iPSCs were triturated into single cells and embryoid bodies (EBs) were formed using AggreWell plate (Stem Cell Technologies, Canada) with N2 medium containing 0.5× N2, 0.5× B27, 1% penicillin/streptomycin in DMEM/F12 medium plus 5 μM Y-27632, 1 μM dorsomorphin, 10 μM SB431542 (Tocris Bioscience, MN). A day later, EBs were transferred to an ultra low attachment petri dish for a 24 hr suspension culture. The next day, EBs were seeded on a matrigel-coated plate using N2 medium for 7–10 days. Rosette-bearing EBs were manually picked and dissociated into individual cells. These dissociated cells were then plated into a poly-l-ornithine/laminin-coated plate to generate a monolayer of NPC culture with N2 medium plus 20 ng/μl FGF2. NPC markers including Sox2 (1:100, Stemgent, MA) and Nestin (1:60, R&D systems, MN) were confirmed positive in these cells using immunocytochemistry.

Generation of astrocytes from NPCs

Astrocytes were differentiated from the NPCs following a protocol detailed elsewhere (Freitas, et al, 2016 Neuron - in press). Briefly, a confluent 100 mm diameter NPC plate was scraped forming neurospheres in a six-well plate by keeping at constant shaking (95 rpm). The media was changed on the day after cells were suspended once the neurospheres were well formed using the NPC media containing FGF. After efficient formation of spheres around 48 hrs post scrapping, the rock inhibitor was added to a final concentration of 5 μM for 48 hrs concomitant with the removal of FGF from the media in the next media change. Cells were kept in constant shaking with neuronal inducing media for a week. Next, the astrocyte growth media (Lonza, Allendale, NJ) was added to the spheres for two weeks still under 95 rpm's. Following two weeks on the astrocyte media, spheres are plated in laminin coated plates and the astrocytes grow out of the sphere spreading on the plate to form a multilayer cell formation. After the first passage, cells surrounding the neurospheres were dissociated enzymatically using accutase (Cellgro) and plated. The neurospheres were removed manually by vacuum suction using a Pasteur pipet and the result was a confluent and homogeneous plate of GFAP and S100β positive astrocytes.

Experimental conditions

Artificial cerebral spinal fluid (ACSF) was balanced with 5% CO_2 + room air. ACSF contained (in mM): NaCl 129, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 1.5, $NaHCO_3$ 21, and glucose 10. The osmolarity was 315 mOsm and pH was 7.4. Ischemic solution (IS) (Yao et al., 2007a) was balanced with 15% CO_2 + 1.5% O_2 + N_2. IS contained (in mM): NaCl 47, KCl 29, K-gluconate 35, $MgCl_2$ 1.5, $CaCl_2$ 0.13, $NaHCO_3$ 4, glucose 3, and glutamate 0.1. The osmolarity was adjusted to 315–320 mOsm with sucrose and pH was 6.4. All of the above chemicals and S0859 were obtained from Sigma.

Intracellular pH measurement

A coverslip with cultured astrocytes was mounted on a thermostatically controlled holding chamber and incubated with 2 μM membrane-permeable ester BCECF-AM for 20 min at room temperature. The chamber was then secured on the stage of the microscope and supplied with ACSF bubbled with 5% CO_2 balanced with air. Intracellular fluorescence was determined in individual cells using fluorescence microscopy and digital image processing. A 175 W Xenon lamp and an ultra high speed wavelength switcher (Lambda DG-4, Sutter Instrument, Novato, CA) provided alternate 440/495 nm fluorescence excitation. The emission from BCECF loaded cells was detected at wavelengths of 535 nm using a F-Fluar 40×/1.3 NA oil immersion objective (Zeiss Axiovert 200M microscope, Zeiss, Yena, Germany) and the attached 12 bit CCD camera. The light source, wavelength switcher, microscope and the camera were controlled by a computer. The ratio images were sampled every 15 seconds. Data were recorded and analyzed with MetaFlour imaging-processing software (Universal Imaging Corporation, Downington, PA). Ratios ($R_{495/440}$) were obtained from these two fluorescence emission intensities and the calibration of pH_i was conducted with the single-point nigericin method (Boyarsky et al., 1988).

Ca^{2+} imaging

Using the imaging system described above, intracellular Ca^{2+} was measured with 2 μM calcium-sensitive fluorescent dye Fluo 4-AM (Molecular Probes, Eugene, OR). Calcium waves were induced by focal mechanical stimulation of single astrocytes in the center of the field of view as described previously (Scemes et al., 1998). The wavelength switcher provided 485 nm fluorescence excitation. The emission from Fluo-4 loaded cells was detected at a wavelength of 520 nm using the oil immersion objective and the CCD camera. Changes in Fluo-4 fluorescence intensities emitted at 520 nm were acquired at 0.67 Hz. The calcium imaging data were visualized and analyzed offline in MetaMorph software.

Real time PCR

RNA was isolated from astrocytes using the RNeasy Mini kit (Qiagen, CA). cDNA was produced from total RNA through RT-PCR using the Superscript III First-Strand Synthesis system (Invitrogen, CA). Real-time PCR was performed using a GeneAmp 7900 sequence detection system using POWER SYBR Green chemistry (Applied Biosystems, CA). Real-time PCR was conducted by unbiased approach by designing primers in the regions that can amplify all the isoforms of NBCe1 or NBCn1. The primer sequences are as follows: NBCe1-L: AGCACCTCACTATCTGAAAGGC; NBCe1-R:

CACAACTTGACTGGTTGGCG; NBCn1-L: AGCCAAGCACACAGTCTTTCT; NBCn1-R: AATGGGATGCCACAGCTTTT. The expression level of GADPH was used to normalize the results. GADPH-L: CTG GCATTGCCCTCAACGACC; GADPH-R: CTTGCTGGGGCTGGTGGTCC.

Western blot analysis

Membrane proteins were isolated using a protein extraction kit (Abcam, MA). Primary antibodies against human NBCe1 (anti-SlC4A4 rabbit polyclonal antibody, diluted 1:500) or NBCn1 (anti-SlC4A7 rabbit polyclonal antibody, diluted 1:500) were obtained from Abcam (Abcam, MA). The secondary antibody (anti-rabbit, diluted 1:1000) was obtained from Invitrogen (Invitrogen, MD). Anti-SlC4A4 antibody reacts to conserved amino acids at N-terminal domain of SLC4A4 and it covers all isoforms of SlC4A4. Similarly, anti-SlC4A7 antibody reacts with all the isoforms of SlC4A7. Western blotting was performed as described elsewhere. Briefly, 20 μg of lysate supernatant was separated by 4–12% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were developed using ECL reagents (Bio-Rad, CA) and the ChemiDoc XRS+ molecular imager from Bio-Rad. GADPH (Santa Cruz Biotechnology, CA) was used as the loading control for all blots.

Hoechst/PI assay

Cells were stained with Hoechst 33342 (10 μg/ml) and propidium iodide (PI) (5 μg/ml) for 10 mins and 24 hrs, respectively, and examined under fluorescence microscopy. Cell death was quantitated by counting cells in 10 randomly chosen fields from two different experiments and the results were expressed by the ratio of PI positive cells to total cells (Hoechst positive cells).

Statistical Analyses

Differences in main effects between two groups were examined with a student t-test. Differences among multiple groups were determined by a two-way ANOVA followed by Bonferroni's post test, using GraphPad Prizm software (GraphPad, San Diego CA). The criterion for statistical significance was a p value of <0.05.

Results

Generation of astrocytes from human iPSCs

Astrocytes were differentiated from hiPSCs as shown in Fig. 1. Neuroprogenitor cells (NPCs) were differentiated from the hiPSCs (Fig. 1A) and were positive for Nestin and SOX2 immunostaining, the typical NPC markers used (Fig. 1B). Cells differentiated from the NPCs were positive for the immunostaining of astrocytic markers, GFAP and S100β (Fig. 1C). Furthermore, to examine if the astrocytes were capable of propagating calcium waves, a phenomenon characteristic to astrocytes, these cells were loaded with a calcium sensitive fluorescent dye Fluo-4 and monitored under a fluorescent video-microscope. During the experiment, one cell was chosen and slightly touched by a glass pipette tube with a tip of 1μm in diameter. The stimulated cell was immediately fluorescent due to increased intracellular calcium and this increase in intracellular calcium was propagated to the neighboring cells gradually and formed a calcium wave among numerous cells (Fig. 1D).

Functional characterization of the NBCe

A HCO_3^--dependent mechanism contributes to the maintenance of steady-state pH_i in astrocytes—Steady-state pH_i (ss pH_i) was measured from astrocytes derived from skin biopsies of two human subjects with an average of 5 min recording either in the absence or presence of CO_2/HCO_3^-. As has been described previously in rodent astrocytes (Bevensee et al., 1997), hiPSCs-derived astrocytes have a broad range of ss pH_i when bathed in a solution in the nominal absence of CO_2/HCO_3^-. The ss pH_i of hiPSCs-derived astrocytes ranged from 6.5 to 7.2, with a mean ss pH_i of 6.85 ± 0.12 (pooled from two human subjects: 6.84 ± 0.13, n=57; 6.86 ± 0.11, n=58) in the absence of CO_2/HCO_3^-. In the presence of CO_2/HCO_3^-, however, the ss pH_i of astrocytes was 7.14 ± 0.24 (pooled from two human subjects: 7.16 ± 0.19, n=60; 7.11 ± 0.28, n=54) which was significantly higher than that in the absence of CO_2/HCO_3^- (Figs. 2A & 2B, $p<0.05$).

The effect of S0859 on ss pH_i in astrocytes—In this experiment, astrocytes were initially incubated in the CO_2/HCO_3^- solution which later was switched to the same solution but supplemented with 30 μM S0859, a selective NBC inhibitor (Ch'en et al., 2008). As shown in Fig. 2C, S0859 elicited a decrease in pH_i which was recovered upon the removal of the inhibitor. Our results show that the S0859 acidified the ss pH_i from 7.13 ± 0.02 to 6.82 ± 0.04 in astrocytes, which is significantly different (n=23, $p<0.05$, Fig. 2D).

Depolarization-induced alkalinization in astrocytes is HCO_3^- dependent—The effect of membrane depolarization on ss pH_i was examined by exposing astrocytes to a 15 mM K^+ in the CO_2/HCO_3^- solution as described previously (Grichtchenko and Chesler, 1994). Depolarization-induced alkalinization (DIA) were recorded as shown in Fig. 2E. The amplitude (ΔpH_i) of the DIA elicited by a 15 mM K^+ was 0.15 ± 0.02 in the presence of CO_2/HCO_3^-, which is significantly higher than 0.02 ± 0.01 in the absence of CO_2/HCO_3^- (n=18, $p<0.05$, Fig. F).

S0859 inhibits the DIA in astrocytes—To examine if the DIA was mediated by NBCe, the DIAs were induced before and during the application of the NBC inhibitor S0859. Fig. 2G shows the trace of two DIAs elicited by 15 mM K^+ application in the same cell. The amplitude of the DIA was significantly smaller when elicited in the presence of a 30 μM S0859. Statistical analysis shows that, in the presence of S0859, a 15 mM K^+ elicited a DIA of 0.04 ± 0.02 pH unit, which is significantly smaller than 0.21 ± 0.03 (n=16, $p<0.05$, Fig. 2H) in the absence of S0859, demonstrating that the S0859 inhibited the DIA induced by high K^+.

IS enhances NBCe1 mRNA and protein expression

We performed qPCR to determine the relative change of mRNA level in NBCe1 (electrogenic Na^+/HCO_3^- co-transporter 1) and in NBCn1 (electroneutral Na^+/HCO_3^- co-transporter 1) following IS treatment. Astrocytes were incubated with IS or ACSF control for 24 hrs and the qPCR results show a 2-fold increase of NCBe1 mRNA in the IS treated vs. in the ACSF-incubated astrocytes (Fig. 3A, n=3 for each group, $p<0.05$). The expression level of the NBCn1 mRNA was not significantly changed after the IS treatment as shown in Fig. 3B (n=3 for each group, $p>0.05$). The NBCe1 and NBCn1 protein expression levels

were also compared between the IS treated and the control group in astrocytes. Western blot analysis shows that a 24-hr IS treatment induced a 3 fold increase in the NBCe1 protein expression level (Fig. 3C, n=3 for each group, $p<0.05$) and no significant change was detected in NBCn1 protein expression level (Fig. 3D, n=3 for each group, $p>0.05$).

IS induces cell death in astrocytes

Astrocytes derived from hiPSCs were treated with IS for 24 or 48 hrs and cell death was assessed by propidium iodide (PI) and Hoechst stains. As shown in Fig. 4A, B & C, IS induced significant amount of cell death in astrocytes. After a 24-hr treatment, there was a 43.6 ± 4.5% (n=10) cell death in the IS-treated group compared with a 6.3 ± 2.5% (n=10, $p<0.001$) cell death in the untreated group (Fig. 4A & B). Cell death increased to 58.4 ± 5.4% (n=10, $p<0.001$, compared with the 24-hr group) when the duration for IS treatment was prolonged to 48 hrs (Fig. 4B & C).

S0859 exaggerates the IS induced cell death in astrocytes

The effect of S0859 on IS-induced cell death was examined using a series of doses of S0859, 0, 0.1, 1, 10, 30, 100 and 1000 μM. Astrocytes was pretreated with S0859 2 hr before the onset of a 24-hr IS+S0859 treatment. As shown in Fig. 4F, in astrocytes incubated in normal culture conditions, at the above dose range of S0859, S0859 did not induce any significant damage to astrocytes until the S0859 reached 1000 μM. In the IS-treated astrocytes, however, cell death increases along with the increased concentration of S0859. For example, in the IS-treated astrocytes, a 10 μM S0859 induced a 62.0±8.3% (n=10) cell death, which was significantly higher than the control group (43.6 ± 4.5%, n=10, $p<0.001$, Fig. B). When the S0859 was increased to 30 μM, cell death was increased to 97.5±1.6% (n=10, Fig. E), which is significantly different from that obtained at 10 μM S0859 ($p<0.001$, Fig. F). While in the control group, astrocytes treated with 30 μM S0859 did not show significant PI uptake and damage (6.6 ± 2.9%, n=10, Fig. 4D) compared with the untreated group (6.3 ± 2.5%, n=10, $p>0.05$, Fig. 4A, F).

The effect of S0859 on IS induced pH_i change in astrocytes

To examine how IS affects the activity of NBC, we conducted the following two experiments. In the first experiment, the effects of a 24-hr IS treatment on the pH_i was examined in both the absence and presence of a 10-min S0859 treatment on the same astrocytes. As shown in Fig. 5A, IS elicited a significant pH_i drop in astrocytes which was stable at a mean level of 6.28 ± 0.03 (n=34). This IS-induced pH_i decrease is significantly different from the ss pH_i (7.11 ± 0.28, n=54, $p<0.05$, Fig. 5C). After obtaining a stable pH_i baseline in IS, the perfusate was switched to the solution supplemented with 30 uM S0859. As shown in Fig. 5A, a 10 min S0859 treatment did not elicit a change in pH_i in the astrocytes. The mean pH_i level during the 10-min S0859 incubation was 6.32 ± 0.05, which is not significantly different from 6.28 ± 0.03 when incubated with IS alone (n=34 from both groups, $p>0.05$), suggesting that the activity of NBCe was already inhibited by IS.

In another experiment, pH_i was measured in astrocytes treated with IS+S0859 for 24 hrs. Fig. 5B shows a representative trace demonstrating a pH_i level with the calibration solution applied at the end of the recording. Statistical analysis shows that the mean pH_i was 6.32

± 0.05 (n=29) in astrocytes following a 24-hr IS+S0859 treatment, which is significantly lower than the baseline pH_i (7.11 ± 0.28, n=54, $p<0.05$, Fig. 5C) in control astrocytes but is similar to 6.28 ± 0.03 (n=34) when astrocytes were incubated with IS alone ($p>0.05$, Fig. 5C), suggesting that the inhibition of NBC by S0859 did not impose a significant further acidification in these astrocytes.

Discussion

In this study, we made the following observations: a) the NBCe1 mRNA and protein expression is elevated in astrocytes treated with IS solution that simulates the ischemic penumbral environment; b) the NBC blocker, S0859, exaggerates the IS-induced cell death in astrocytes and c) the mechanism underlying the exaggeration of IS-induced astrocyte death by blocking NBC is independent of its role in pH_i regulation.

Bicarbonate-dependent acid base transporters, especially NBCe1, have been previously studied in astrocytes, mainly in rodents and other non-human species, although a few of these studies have used human astrocytic cell lines (Majumdar et al., 2008, Ruminot et al., 2011, Theparambil et al., 2015). Our current study shows a similar ss pH_i of 6.85 in hiPSCs-derived astrocytes to that of rodents: in cultured rat cortical astrocytes, the ss pH_i is 6.83±0.01 (Bevensee et al., 1997), while the ss pH_i is 6.82±0.06 in cultured mouse cortical astrocytes (Hansen et al., 2015). hiPSCs-derived astrocytes also propagate calcium waves as reported previously in rodents (Charles, 1998). Therefore, we believe that results obtained from hiPSCs-derived astrocytes are a better *in vitro* model than animal astrocytes in the research field of human physiology, pathophysiology and pharmacology.

Astrocytes express both electrogenic and electroneutral NBCs in mammalian brain and the electrogenic NBC plays a major role in some pathological states (Giffard et al., 2000, Schmitt et al., 2000, Theparambil et al., 2014, Schrodl-Haussel et al., 2015). Recent work has demonstrated the alteration of the expression profile of NBCe under ischemia conditions. In an *in vivo* permanent MCAO model, the expression of the NBCe mRNA and protein was found to be significantly elevated in the ischemic penumbral tissue (Jung et al., 2007). Although the cell type of these NBCe-expressing cells was not specified, we believe that astrocytes were among them. Indeed, using the ischemic solution that simulates the ischemic penumbral environment, our current work has demonstrated a significant elevation of the NBCe1 mRNA and protein levels in astrocytes following a 24-hr exposure to ischemic penumbral environment. Our data are consistent with the above observation made *in vivo* and suggest that the changed microenvironment in the ischemic penumbral tissue triggers the NBCe1 expression to provide astrocytes with an increased level of NBCe1 for protection. In contrast, our data show that IS did not alter the expression of both mRNA and protein of NBCn1, another isoform in the NBC family, indicating some specificity for NBCe1 in ischemic brain injury.

Our major finding in this work is the exaggeration of astrocyte ischemic death by inhibiting the activity of NBC. We have shown in our previous work that the unselective inhibition of the HCO_3^- dependent transporters or the NBCe alter the outcome of ischemia induced astrocyte damage. For example, increased extracellular HCO_3^- attenuates ischemic cell

death, while decreased extracellular K^+ worsens ischemic cell death in astrocytes in organotypic hippocampal slice cultures (Yao et al., 2007b). These data support the notion that factors that enhance NBCe activity is beneficial while attenuating NBCe activity is detrimental to astrocytes in the ischemia penumbral tissue. Indeed, our current data further confirm that blocking the activity of NBC, most likely that of NBCe1, is detrimental to the penumbral astrocytes. Nevertheless, since S0859 is a generic NBC blocker, we cannot rule out the participation of NBCn1 in the ischemic astrocyte death in our work, although our qPCR and Western blot data show that the IS treatment did not alter the NBCn1 mRNA and protein levels.

While our current work shows that NBC inhibition worsens the IS-induced cell death in astrocytes, the mechanism of this process is not immediately clear. Since the failure of pH_i regulation in astrocytes can lead to severe acidosis that damages astrocytes during brain ischemia (Chesler, 2005), the exaggeration of IS-induced astrocytes death by S0859 could be due to the exacerbation of acidosis induced by IS. However, our data show that the pH_i regulatory activity of NBC is inhibited in astrocytes during ischemia, and the addition of S0859 did not worsen the acidosis induced by IS. Similarly, the 30 μM S0859 treatment used in our experiments could also blunt the pH_i regulatory activity of monocarboxylate transporters (MCTs), such as MCT1 and/or MCT4 in astrocytes (Heidtmann et al., 2015, Hong et al., 2015), suggesting the repression of MCTs by IS. Therefore, it is tempting to speculate that the ion transporting activity related to pH_i regulation does not play a significant role in the S0859 exaggeration of ischemic astrocytes death. Indeed, recent work has suggested that NBC may participate in activating apoptosis pathway in mitochondria. In rat coronary endothelial cells, for example, NBCn1 is co-localized with mitochondria and suppresses the ischemia-induced activation of the mitochondrial pathway of apoptosis (Kumar et al., 2011). Hence, we believe that NBCe may also mediate cell death via signaling pathways that might not be related to pH_i regulation in astrocytes under ischemia.

Acknowledgments

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Abbreviations used

NBC	Sodium bicarbonate co-transporter
NBCe	Electrogenic sodium bicarbonate co-transporter
NBCn	Electroneutral sodium bicarbonate co-transporter
hiPSCs	human induced pluripotent stem cells
NPCs	neural progenitor cells
IS	ischemic solution
BCECF	2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein
PI	propidium iodide

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Highlights

- Generation of astrocytes from human induced pluripotent stem cells (hiPSCs).
- Functional sodium bicarbonate co-transporters (NBCs) exist in hiPSC-derived astrocytes
- Gene expression of electrogenic sodium bicarbonate co-transporter is enhanced under ischemia
- An NBC inhibitor exaggerates Ischemia-induced cell death in hiPSC-derived astrocytes

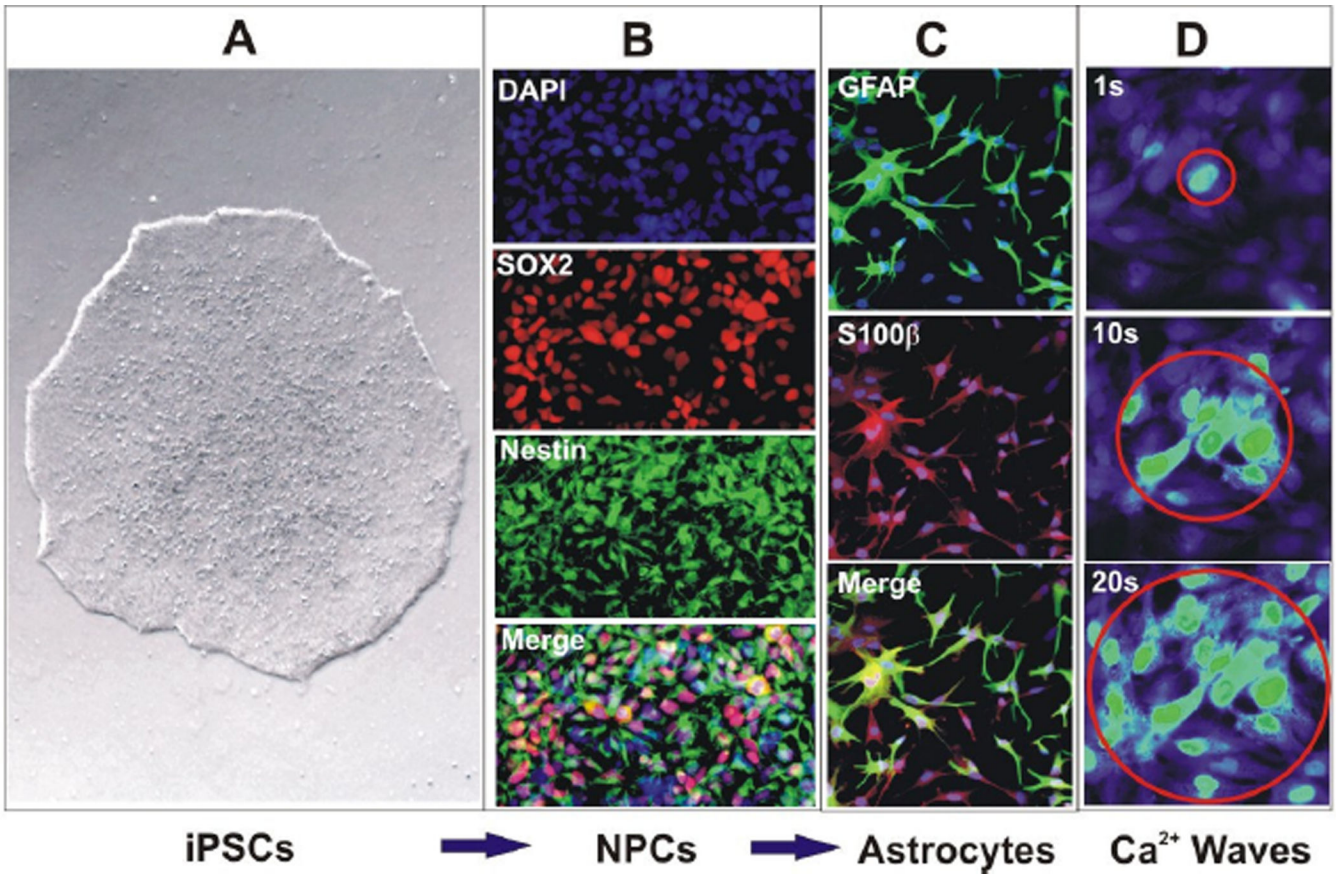

Fig. 1.

Generation of astrocytes from hiPSCs. A multi-step protocol was used to generate astrocytes. From left to right: iPSCs (column A) reprogrammed from skin biopsy of a healthy subject were initially differentiated into NPCs (expressing SOX2 and Nestin shown in column B), which then further differentiated into astrocytes. As shown in column C, the hiPSCs-derived astrocytes express GFAP and S100β. Astrocytes generated from this protocol exhibited a typical astrocyte morphology and calcium waves could be propagated as shown in column D (labels on the top left of each image indicate the time in seconds after the mechanical stimulation of the first cell in the red circle).

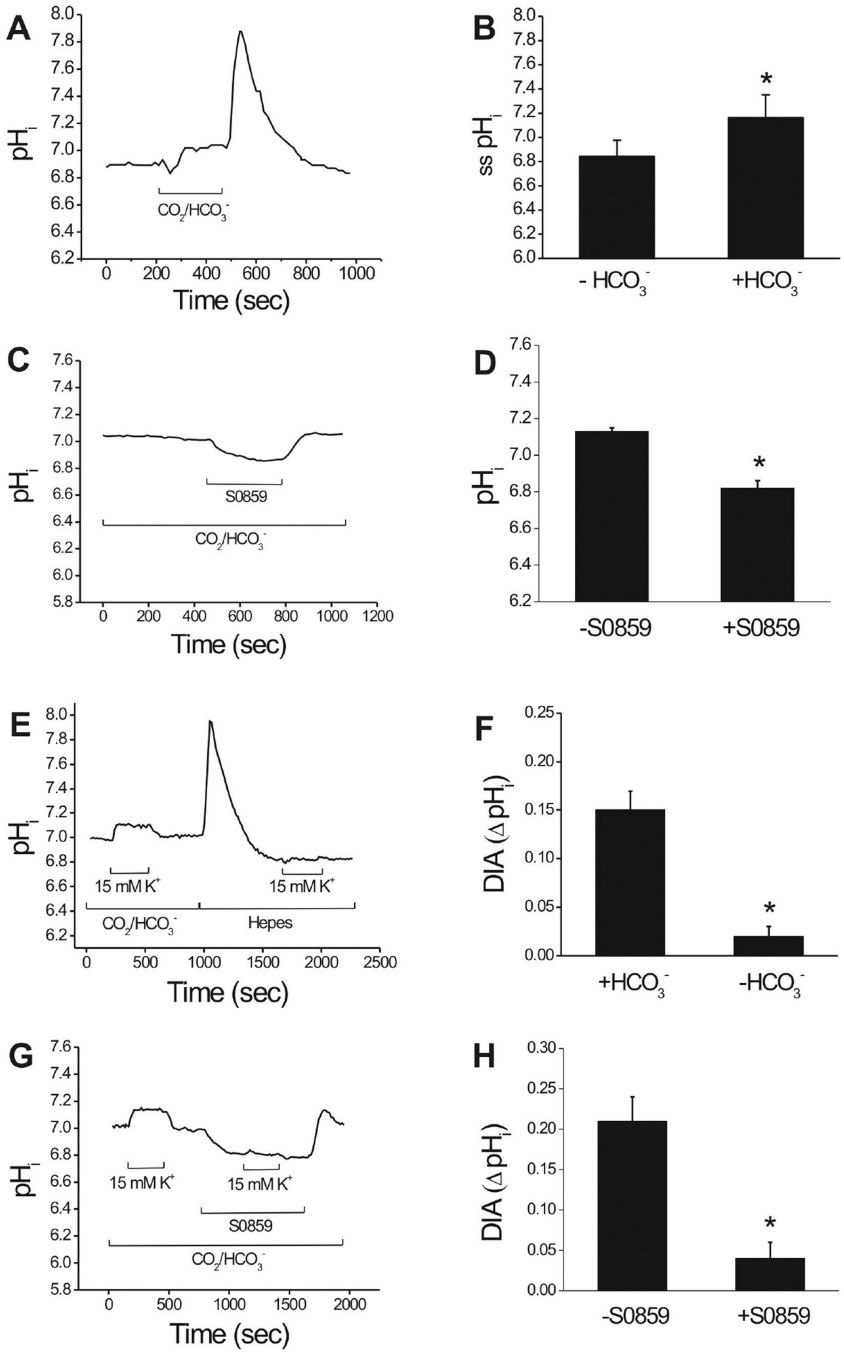

Fig. 2.

Characterization of NBCe in hiPSC-derived astrocytes. A, a representative trace showing a typical pH_i change when bath solution was switched between in the presence or absence of CO_2/HCO_3^-. B, bar graph showing the comparison of ss pH_i in astrocytes between in the presence or absence of CO_2/HCO_3^- in the bath solution (*: $p<0.05$). C, a representative trace showing the pH_i drop elicited by S0859. D, bar graph showing the comparison of ss pH_i in astrocytes incubated in the presence or absence of S0859 (*: $p<0.05$). E, a representative trace showing that the DIA was induced in astrocytes by 15 mM K^+ in the presence but not

in the absence of CO_2/HCO_3^- in the bath solution. F, bar graph showing the comparison of DIA amplitude between in the presence or absence of CO_2/HCO_3^- in the bath solution (*: $p<0.05$). G, a representative trace showing that a 15 mM K^+ induced a DIA which was inhibited by S0859. H, bar graph showing the comparison of DIA amplitude in the presence or absence of the addition of S0859 in the bath solution (*: $p<0.05$).

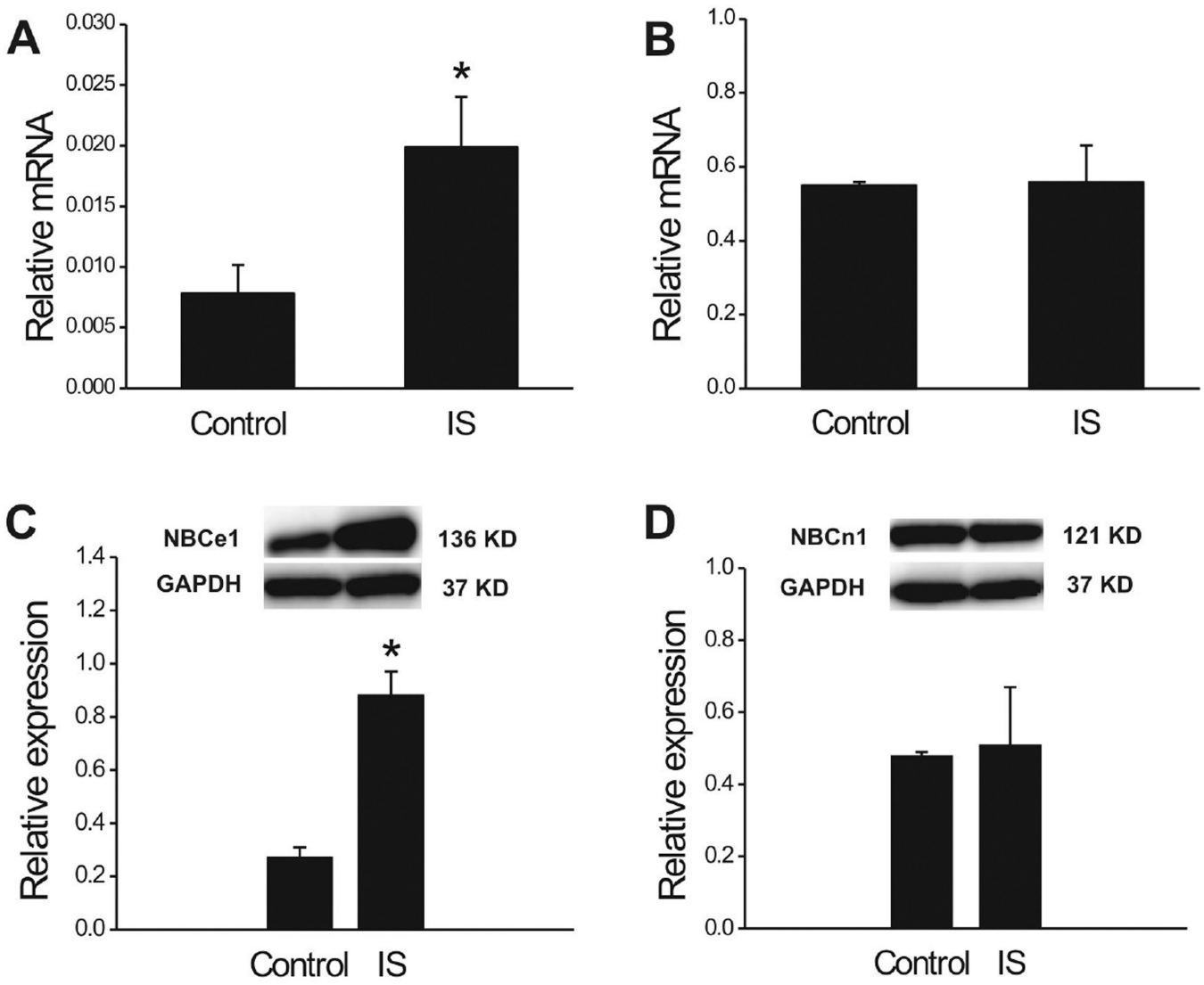

Fig. 3.

The effect of IS on NBCe1 and NBCn1 mRNA and protein expression in astrocytes. A & B, relative mRNA levels of NBCe1 (A) and NBCn1 (B) in astrocytes with and without a 24-hr IS treatment as measured by qPCR (normalized to GADPH, *:$p<0.05$). C & D) Western blot analysis showing the relative quantification of NBCe1 (C) and NBCn1 (D) protein expression levels in astrocytes with and without a 24-hr IS treatment (*: $P < 0.01$).

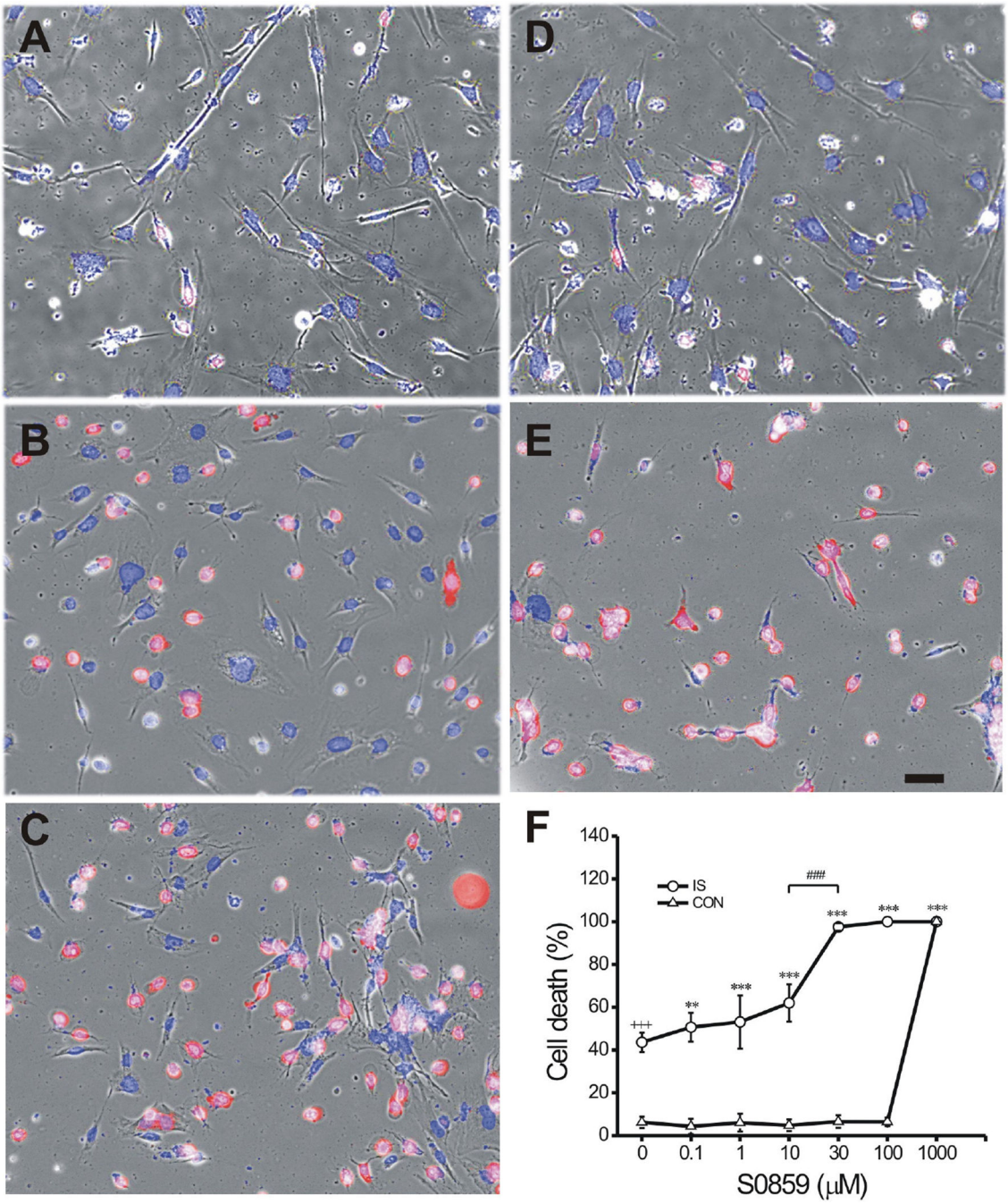

Fig. 4.

S0859 exaggerates IS-induced cell death in astrocytes. A-C, IS induces astrocytes death in a time dependent manor. Overlapped images showing astrocytes (bright field) were stained with blue (Hoechst) and the nucleus of damaged or dead cells were stained with red (propidium iodide). A, representative image showing cells before the IS treatment. B, cells were imaged following a 24-hr IS treatment. C, cells were imaged following a 48-hr IS treatment. D&E, S0859 (30 μM) exaggerates cell damage in astrocytes subjected to a 24-hr IS treatment. D, astrocytes imaged following a 24-hr S0859 (30 μM) incubation without the

IS treatment. E, astrocytes imaged following a 24-hr IS + 30 μM S0859 treatment. Scale bar: 20 μm. F, dose dependent effect of S0859 on IS-induced cell death in astrocytes. +: $p<0.001$, compared between IS and control group before S0859 treatment. *: $p<0.001$, compared between IS and control group with S0859 treatment. #: $p<0.001$, compared between 10 and 30 μM S0859 treatment in IS treated groups.

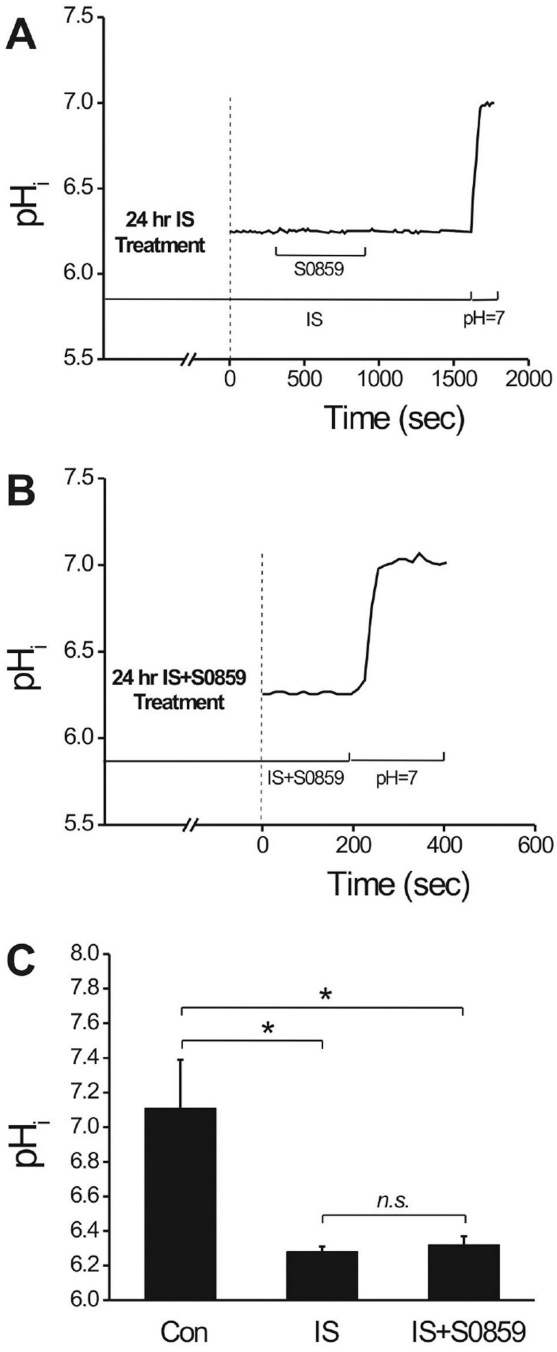

Fig. 5.

The effects of S0859 on IS-induced pH_i change in astrocytes. A, a representative trace showing pH_i measurement in an astrocyte after a 24-hr IS treatment. Note a 10-mins S0859 (30 μM) did not elicit significant change in pH_i in this cell. B, a representative trace showing pH_i measurement in an astrocyte after a 24-hr IS+S0859 treatment. C, bar graph showing the comparison of pH_i levels among groups. Note that both IS and IS+S0859 indicate that

astrocytes were treated with that condition for 24 hrs before conducting pH_i measurement (*: $p<0.05$; *n.s.*: non-significant).