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Acid-Sensing Ion Channel 1a Regulates Fate of Rat Nucleus Pulposus Cells in Acid Stimulus Through Endoplasmic Reticulum Stress

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Abstract

Acid-sensing ion channel 1a (ASIC1a) participates in human intervertebral disc degeneration (IVDD) and regulates the destiny of nucleus pulposus cells (NPCs) in acid stimulus. However, the mechanism of ASIC1a activation and its downstream pathway remain unclear. Endoplasmic reticulum (ER) stress also participates in the acid-induced apoptosis of NPCs. The main purpose of this study was to investigate whether there is any connection between ASIC1a and ER stress in an acid-induced nucleus pulposus degeneration model. The IVDs of Sprague-Dawley rats were stained by immunohistochemical staining to evaluate the expression of ASIC1a in normal and degenerated rat nucleus pulposus. ASIC1a expression was also quantified by quantitative real-time-polymerase chain reaction and Western blotting analysis. NPCs were exposed to the culture media with acidity at pH 7.2 and 6.5 for 24 h, with or without 4-phenylbutyrate (4-PBA, a blocker of the ER stress pathway). Cell apoptosis was examined by Annexin V/Propidium Iodide (PI) staining and was quantified using flow cytometry analysis. ASIC1a-mediated intracellular calcium was determined by Ca^{2+} imaging using Fura-2-AM. Acidity-induced changes in ER stress markers were studied using Western blotting analysis. *In vivo*, ASIC1a expression was upregulated in natural degeneration. *In vitro*, acid stimulus increased intracellular calcium levels, but this effect was blocked by knock-down of ASIC1a, and this reversal was partly inhibited by 4-PBA. In addition, blockade of ASIC1a reduced expression of ER stress markers, especially the proapoptotic markers. ASIC1a partly regulates ER stress and promotes apoptosis of NPCs under acid stimulus and may be a novel therapeutic target in IVDD.

Keywords: acid-sensing ion channel 1a; apoptosis; endoplasmic reticulum stress; intervertebral disc degeneration; nucleus pulposus

Background

Low back pain is strongly associated with intervertebral disc degeneration (IVDD) and imposes significant social and economic burdens.^{1–3}

Within the anaerobic environment of the intervertebral disc (IVD), nucleus pulposus cells (NPCs) undertake glycolysis depending on their endoplasmic reticulum (ER) function, and the IVD is thus characterized by high concentrations of lactic acid and low pH.⁴ Lactic acid, in turn,

triggers ER stress by disrupting protein folding and calcium homeostasis.^{5–7} Its protein maturation machinery and other energy-intensive processes make the ER highly sensitive to numerous factors, including toxins, nutrition, and energy deprivation, redox imbalances, and disorder in Ca^{2+} storage.⁸

As members of the degenerin/epithelial Na^+ channel superfamily, acid-sensing ion channels (ASICs) are H^+ gated voltage insensitive. To date, six ASIC subunits

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have been identified—ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4—and are encoded by four ASIC genes.⁹ Homomeric ASIC1a is Ca^{2+} and Na^{+} permeable, whereas other subunit combinations are only Na^{+} permeable. The pH values required for half-maximal activation of ASICs ($\text{pH}_{0.5}$) vary from 4.9 to 6.8.¹⁰

According to results of our previous study,⁵ activation of Ca^{2+} -permeable ASIC1a participates in IVDD and regulates the destiny of human NPCs in acid stimulus. We have also demonstrated that ER stress participates in the survival of cells during acid-induced NPCs apoptosis.⁶ However, it remains unclear if ASIC1a directly mediates Ca^{2+} influx and increased intracellular Ca^{2+} [Ca^{2+}]_i in rat NPCs. Direct evidence for a causal role of ASIC1a-mediated Ca^{2+} entry in ER stress is lacking.

In the present study, we tested the hypothesis that extracellular acidosis increases NPCs apoptosis through ASIC1a-mediated calcium entry and the subsequent activation of ER stress. The main purpose of this study was to determine the relationship between ASIC1a and ER stress in NPCs under acid stimulus.

Materials and Methods

All procedures specified below were approved by the Institutional Animal Care and Use Committee of Southeast University (Nanjing, China, 2016ZDKYSB089).

In vivo studies

Sprague-Dawley rats (24 males, age 8–40 weeks) were euthanized with CO_2 and were examined by magnetic resonance imaging (MRI, 7T; Bruker, Germany) to evaluate the level of degeneration. Following imaging, the spine was collected from midthoracic to lumbar segments. After fixation, decalcification, and embedding in paraffin, coronal sections 3 μm in thickness were prepared according to methods described in our previous study.¹¹ The sections were stained with the conventional Hematoxylin and Eosin (H&E) staining method.

Immunohistochemical and immunofluorescence staining

Deparaffinized coronal sections were blocked with 5% bovine serum albumin (BSA) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween20) and were incubated with primary antibodies at 4°C overnight. The sections were subsequently incubated with secondary antibody diluted in phosphate-buffered saline (PBS) (A0208, 1:5000, Beyotime, China; Alexa Fluor 594, 1:1000, Invitrogen) for 1.5 h at room temperature. Immunohistochemical stain-

ing was viewed with light microscopy (Olympus BX41, Japan). For immunofluorescence staining, 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Ireland) was used to counterstain cell nuclei for 5 min at room temperature, and the cells were observed with a fluorescence microscope (Olympus, Japan). For the negative control, the same procedure was performed, omitting incubation with primary antibodies.

In vitro studies

Isolation and culture of NPCs. Sprague-Dawley rats (20 males, age 8–40 weeks) were euthanized with CO_2 . NP tissues were collected as previously described.^{11,12} The incompletely digested NP was maintained in DMEM/F-12 (GIBCO, Grand Island, NYA) containing 10% FBS (Elite Biotech, Germany) and 1% penicillin/streptomycin (GIBCO) and cultured in 95% humidity, 21% O_2 , and 5% CO_2 at 37°C. Confluent cells were lifted using 0.25% Trypsin-EDTA (1×) solution (Life Technologies, Canada) and were then subcultured (1:2) to collect passage 1 (P1) and P2 cells. The P2 NPCs were used in the subsequent assays.

RNA interference of ASIC1a. We used retroviral vectors containing ASIC1a-siRNA (constructed by Hanbio, China), and the retrovirus-expressing blank served as a negative control. A monolayer of NPCs with 70% confluence were infected with the retrovirus-containing supernatant in the presence of polybrene (6 $\mu\text{g}/\text{mL}$, Hanbio) for 24 h, and then were exposed to puromycin (2 $\mu\text{g}/\text{mL}$, Hanbio). Western blot analysis was performed to evaluate levels of RNA interference. At 5 days after infection, cells were subjected to acid treatment.

Exposure of NPCs to acid. As described in our previous studies,^{5,6} pH 6.5 was selected as a mimic of degenerated IVD, and pH 7.2 was used as the control. Cells were incubated with 4-phenylbutyrate (4-PBA) (5 mM; Sigma) for 24 h before acid treatment. The cells were then harvested after exposure to the acidic condition.

Detection of activation of ASIC1a by Fura-2-AM. As described in our previous studies, cells on glass bottom dishes (Cellvis) were washed with PBS twice and then incubated with Fura-2-AM (5 μM ; Sigma, Ireland) for 30 min at room temperature. After washing twice again, Fura-2-AM fluorescence was observed with a laser scanning confocal microscope (Olympus) with excitation at 488 nm and emission at 525 nm. Observation

continued when the acidic solution (pH 6.5) was added and continued for 10 min thereafter. The mean fluorescence value was calculated and the intensity of the fluorescence was measured by LSM 5 Image software.

Apoptotic analyses by flow cytometry. Apoptosis analyses were performed using the Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Dual-Staining Kit according to the manufacturer's instructions (KeyGen, China). Floating and attached cells were collected, resuspended in 500 μ L binding buffer, and incubated with 5 μ L Annexin V-FITC and 5 μ L PI for 10 min at room temperature in the dark. Cells were quantified by flow cytometry (BD Accuri C6).

Quantitative real-time polymerase chain reaction. NPCs were collected, and total mRNA was extracted using the Universal RNA Extraction Kit (9767; TaKaRa, Dalian, China). Reverse transcription was performed by following the manufacturer's instructions for the PrimeScript RT Reagent Kit with gDNA Eraser (RR047; TaKaRa), and real-time polymerase chain reaction (RT-PCR) was performed using SYBR Green PCR Master Mix (Roche Applied Science) in a StepOnePlus PCR System (Applied Biosystems). Gene-specific primer sequences are listed in Table 1.

Protein extraction and Western blotting. As in our previous research,^{11,13} total proteins were extracted using whole-cell and tissue lysis assay (KeyGen). Protein quantification using a BCA assay (Beyotime) was performed according to the manufacturer's instructions. Total proteins were resolved on 4–20% Bis-Tris gels (GenScript, China) and transferred by electroblotting onto nitrocellulose membranes (Pall). The membranes were blocked with 5% nonfat dry milk in TBST for 1.5 h and incubated overnight at 4°C with primary antibodies (Table 2). After washing with TBST, the membranes were incubated for 1 h with secondary antibodies (ab6721, 1:5000; Abcam) diluted in 5% BSA/TBST at room temperature. Super Signal

Table 2. Antibodies Used in Western Blotting and Immunohistochemistry

Target	Antibody (Company, LotID, usage)
<i>ASIC1a</i>	Alomone Labs, ASC-014 (1:200)
<i>GRP78 (BIP)</i>	Abcam, ab21685 (1:1000)
<i>XBP1</i>	Abcam, ab37152 (1:500)
<i>CHOP (DDIT3)</i>	Abcam, ab10444 (1:250)
<i>Caspase12</i>	Abcam, ab62484 (1:500)
<i>p-eif2α</i>	Abcam, ab32157 (1:500)
<i>t-eif2α</i>	Abcam, ab131495 (1:500)
<i>Vinculin</i>	Abcam, ab129002(1:10000)

West FEMO Chemiluminescent Substrate (Thermo Scientific) was used for detection. The protein expression level was determined by densitometric analysis and normalized to the level of vinculin.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. All data are expressed as the mean $\pm$ SD and were taken from multiple independent experiments. The results were compared among different groups by using one-way ANOVA and the unpaired *t*-test. *p*-Values <0.05 were considered as significant.

Results

Morphology of rat nucleus pulposus

H&E staining and MRI were utilized to evaluate the basic morphology of rat intervertebral discs. During the degenerative process, the number of NPCs decreased as the moisture content decreased (Fig. 1A, C).

Expression of ASIC1a in rat NP

ASIC1a expression in rats was determined using immunofluorescence and immunohistochemical analyses (Fig. 1B). ASIC1a expression was measured in healthy and degenerated IVDs by Western blot analysis and quantitative RT-PCR (Fig. 1D). In comparison with the healthy NP, expression of ASIC1a was significantly higher in the setting of natural degeneration, both in tissue and at cellular levels ($p < 0.01$, $n = 10$).

Activation of ASIC1a in rat NPCs under acid stimulus

ASIC1a-siRNA was used to knock down *ASIC1a* of rat NPCs, and the level of interference was quantified by Western blot analysis (Fig. 2A).

To evaluate acid-induced activation of ASIC1a of NPCs, the $[Ca^{2+}]_i$ in NPCs was examined by Fura-2-AM fluorescent Ca^{2+} imaging after treatment with extracellular acid. Ca^{2+} imaging revealed a significant increase in intracellular Ca^{2+} in response to an acid

Table 1. Gene-Specific Primer Sequences

Gene	Primers
<i>ASIC1a</i>	
Forward	CACAGATGGCTGATGAAAAGCAG
Reverse	CATGGTAACAGCATTGCAGGTGC
β -actin	
Forward	CCCATCTATGA GGGTTACGC
Reverse	TTTAATGTCACGCACGATTTC

FIG. 1. (A) H/E staining of NP sections of healthy and degenerated rat intervertebral disc. NPCs were sparse in healthy NP with vacuoles inside, which disappeared during senescence. In the degenerated NP, loosened NP tissues were replaced by fibrous tissues and the remaining NPCs reunited into several cell masses. (B, C) Immunofluorescent and immunohistochemical staining of healthy and degenerated rat intervertebral discs. ASIC1a expression increased in the degenerated disc. (D, E) Western blot analysis and quantitative RT-PCR of ASIC1a. Compared with the healthy NP, ASIC1a expression was significantly higher in degenerated NP. ** $p < 0.01$. NPCs, nucleus pulposus cells.

stimulus (pH 6.5) containing Ca^{2+} , and knockdown of ASIC1a inhibited acid-stimulated increase in $[\text{Ca}^{2+}]_i$ in rat NPCs (Fig. 2B, C).

The results of Annexin V-FITC/PI double-staining also showed that the percentage of apoptotic cells significantly increased at pH 6.5 compared with that at pH 7.2. The fraction of apoptotic NPCs significantly decreased when ASIC1a was knocked down by siRNA (Fig. 2D, E).

Acid-induced activation of ASIC1a regulated ER stress in NPCs

To examine the correlation between activation of ASIC1a and acid-induced ER stress in NPCs, Western blot analysis was performed to quantify the expression of ER stress markers. GRP78 was selected as the initiation of ER stress, XBP1 spliced/XBP1unspliced and phosphorylation of eIF2 α were taken as markers of unfolded protein response (UPR), and Caspase12 and

FIG. 2. (A) Compared with the two control groups, expression of ASIC1a was notably downregulated in the siASIC1a group. (B) Ca^{2+} imaging demonstrated that intracellular Ca^{2+} concentration significantly increased in response to the acidic extracellular solution (acid) containing Ca^{2+} . (C) Ca^{2+} imaging indicated by Fura-2/AM at the indicated time. Acidic incubation (pH 6.5) failed to increase $[Ca^{2+}]_i$ in the siASIC1a cells. (D, E) NPCs were incubated with acidity of pH 7.2 or 6.5 for 24 h (flow cytometry). Apoptotic cells = early apoptotic cells (lower right) + late apoptotic cells (upper right). Acid exposure induced apoptosis of rat NPCs. The ER stress inhibitor 4-PBA significantly promoted the apoptosis rate of NPCs under acid stimulus. ASIC1a knockdown reduced acid-induced apoptosis of NPCs, even in the presence of 4-PBA. ** $p < 0.01$. ER, endoplasmic reticulum. 4-PBA, 4-phenylbutyrate.

CHOP were chosen as makers of ER stress-specific apoptosis. This analysis revealed that ER stress markers were upregulated along with acid-induced activation of ASIC1a, and blocking ASIC1a decreased the expression of ER stress markers, especially the proapoptotic CHOP and caspase12 (Fig. 3). The UPR-related marker XBP1 was not affected.

Discussion

The acidity in the NP environment is always a vital factor both in IVDD and biological regeneration. The pH of healthy NP ranges between 7.2 and 7.0.¹⁴ In a mildly degenerative state, the matrix acidity can drop to pH values of 6.8 to 6.5. In the severe stage, the pH can

reach 6.0 and even 5.7. Such pH values are suitable for the activation of ASICs.^{15–17}

Cellular membranes have various Ca^{2+} -transport and Ca^{2+} -binding molecules that participate in Ca^{2+} movement into and out of cells and cellular organelles. Recent studies demonstrated a significant increase in ASIC1 and ASIC4-positive cells in the annulus fibrosus of degenerated IVD and marked upregulation of ASIC1, ASIC2, and ASIC3 in the nucleus pulposus.¹⁸ In addition, it also demonstrated that acid-induced $[Ca^{2+}]_i$ elevation through ASIC1a is involved in endplate chondrocyte apoptosis.¹⁹ To the best of our knowledge, this is the first study to connect ASIC1a with ER stress.

FIG. 3. Compared with the control group, acid triggered expression of ER stress markers, and 4-PBA reduced acid-induced ER stress marker levels. Knockdown of ASIC1a, which had little impact on phosphorylation of eIF2 α and splice of XBP1, significantly downregulated GRP78, CHOP, and Caspase12 in acid stimulus. * $p < 0.05$, ** $p < 0.01$.

In this study, we proved the expression of ASIC1a in healthy and degenerated rat NPCs and investigated the role that ASIC1a plays in acidity-mediated Ca^{2+} influx. The results showed significantly higher ASIC1a expression in degenerated rat discs, at both the RNA and protein levels. We also examined the function of ASIC1a in degenerated rat NPCs. Ca^{2+} influx was significantly reduced in ASIC1a knockdown cells and in Ca^{2+} -free extracellular solution, suggesting that ASIC1a in the cell membrane functions as a Ca^{2+} influx channel in rat NPCs. We previously discovered that even a transient Ca^{2+} signal overload activates a cascade of cytotoxic responses, resulting in the long-term activation of mitochondrial metabolism, and contributing to cellular apoptosis.⁵ An important and well-known aspect of cellular Ca^{2+} signaling is maintaining a balance between free versus bound Ca^{2+} in all cellular compartments. An imbalance in cellular calcium results in Ca^{2+} transport between ER-mitochondria contact sites, causing cellular stress. Such disruption of Ca^{2+} homeostasis in the ER leads to activation of ER stress responses, one of which is the UPR (Fig. 4).

A complex stress response program, such as that in ER stress, always acts in both directions in cellular

stress, acting as a prosurvival response in mild stages and as a proapoptotic response in the prolonged stage. In a previous study, we revealed that ER stress was triggered in NP cell degeneration, primarily playing an antiapoptotic role in acid-induced NP cell apoptosis. Nevertheless, the mechanism of ER stress and transformation has been unclear. In the present study, we found that ASIC1a-activated Ca^{2+} influx is one of the initiation factors of ER stress and is associated with proapoptotic ER stress pathways. We chose to use 4-PBA (a classical blocker of ER stress) in our experiments. While 4-PBA disturbs the recognition of unfolded proteins by molecular chaperon, it decreases the detachment of GRP78 and the downstream UPR pathway. Compared with 4-PBA intervention, blockade of the ASIC1a pathway significantly reduced the level of GRP78, a specific marker of ER stress activation. The multifunctional nature of the ER enables the ER membrane to modulate its own luminal Ca^{2+} dynamics and generate appropriate signals to maintain balanced homeostasis.²⁰ GRP78 (or BiP) is an especially important Ca^{2+} -binding protein, and Ca^{2+} buffer is involved in sensing accumulation of misfolded

FIG. 4. Schematic diagram highlighting ASIC1a and UPR signaling. ASIC, acid-sensing ion channel; ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; ERAD, ER-associated degradation; GRP78, glucose-regulated protein 78; IRE, inositol-requiring enzyme; PERK, protein kinase-like ER kinase; UPR, unfolded protein response.

proteins in the ER. In addition, GRP78 may act as a joint node of ASIC1a and acid-induced ER stress.

CHOP and Caspase12, which are key proteins in ER stress-associated apoptosis, were also considerably down-regulated, whereas the UPR-related ratio of XBP1s/XBP1u and the phosphorylation of eIF2α barely decreased. These findings suggest that acid-induced activation of Ca²⁺-permeable ASIC1a predominantly affects the proapoptotic ER stress pathway and that the UPR maintains the homeostasis of the internal environment. Flow cytometry also demonstrated that knockdown of ASIC1a markedly reduces acid-induced NPC apoptosis, and also suggests that ASIC1a would be a good target to reduce NPC apoptosis in an acidic environment.

The present study has several limitations. First, as a preliminary study, we only demonstrated that the activation of ASIC1a is involved in rat NPC apoptosis and it may play a role in acidosis-mediated ER stress. Although this study provides a novel insight into IVD biology, additional research is necessary to strengthen this finding. Second, ASIC1a is not the only Ca²⁺ channel in the cell membrane that is acti-

vated in acidic environments, and intracellular calcium is not the only initiator of ER stress. In our experiments, we also found that the expression of GRP78 was markedly upregulated when we incubated siASIC1a-NPCs with 4-PBA in acidic environments, suggesting that some other cellular pathways had been activated to initiate ER stress. Third, we examined ER stress from a functional perspective with some representative markers. The temporal sequence of ER stress activation needs to be further examined to better understand ER stress and calcium homeostasis. Fourth, besides ASIC1a, ASIC3 was also found to be upregulated in acid-induced cell stress in our study. As reported,^{15,16,21} ASIC3 may be a cofactor in acid stimulation and calcium influx, and it has also been described as being involved in the expression of proinflammatory cytokines,¹⁷ but further studies are required to substantiate this idea.

Conclusion

Findings of this study support an essential role for ASIC1a in acid-induced NPC death. Activation of ASIC1a and

the ASIC1a-mediated ER stress pathway primarily participates in NPCs apoptosis. ASIC1a may also be a prospective target for treating NP degeneration.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

4-PBA = 4-phenylbutyrate
ASICs = acid-sensing ion channels
ATF6 = activating transcription factor 6
BiP = binding immunoglobulin protein
BSA = bovine serum albumin
DAPI = 4,6-diamidino-2-phenylindole
ER = endoplasmic reticulum
ERAD = ER-associated degradation
FITC = fluorescein isothiocyanate
GRP78 = glucose-regulated protein 78
IRE = inositol-requiring enzyme
IVDD = human intervertebral disc degeneration
MRI = magnetic resonance imaging
NC = negative control
NPCs = nucleus pulposus cells
PERK = protein kinase-like ER kinase
PI = propidium iodide
RT-PCR = real-time-polymerase chain reaction
UPR = unfolded protein response

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