Expression of acid-sensing ion channels in nucleus pulposus cells of the human intervertebral disk is regulated by non-steroid anti-inflammatory drugs

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Non-steroid anti-inflammatory drugs (NSAIDs) are generally used in the treatment of inflammation and pain through cyclooxygenase (COX) inhibition. Mounting evidence has indicated additional COX-independent targets for NSAIDs including acid-sensing ion channels (ASICs) 1a and 3. However, detailed function and mechanism of ASICs still remain largely elusive. In this study, the impact of NSAIDs on ASICs in nucleus pulposus cells of the human intervertebral disk was investigated. Nucleus pulposus cells were isolated and cultured from protruded disk tissues of 40 patients. It was shown that ASIC1a and ASIC3 were expressed and functional in these cells by analyzing proton-gated currents after ASIC inhibition. We further investigated the neuroprotective capacity of ibuprofen (a COX inhibitor), psalmotoxin-1 (PcTX1, a tarantula toxin specific for homomeric ASIC1a), and amiloride (a classic inhibitor of the epithelial sodium channel ENaC/DEG family to which ASICs belong). PcTX1-containing venom has been shown to be comparable with amiloride in its neuroprotective features in rodent models of ischemia. Taken together, our data showed that amiloride, PcTX1, and ibuprofen decreased ASIC protein expression and thereby exerted protective effects from ASIC inhibition-mediated cell damage.

Keywords acid-sensing ion channel (ASIC); intervertebral disk nucleus pulposus cells; non-steroid anti-inflammatory drug (NSAID); ibuprofen

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Introduction

Dorsal disk prolapse remains to be one of the most disabling medical diseases, and existing treatments are often ineffective. Although current therapies reduce dorsal disk prolapse, they fail to resolve symptoms completely. The intervertebral disk is a specialized biomechanical structure that permits motion between vertebrae and absorbs mechanical loads [1]. The disk is bounded by the end plate cartilages of adjacent vertebrae and a circumferential ligamentous structure, the annulus fibrosus. Contained within the disk are cells of the nucleus pulposus; these cells secrete matrix macromolecules, which regulate osmotic pressure, to serve to accommodate applied mechanical forces. The nucleus pulposus cells generate almost all of their metabolic energy through the glycolytic pathway [2]. One reason for the reliance on anaerobic metabolism is that the disk pH is low. How nucleus pulposus cells adapt to and function in an acidotic and hyperosmotic microenvironmental niche is still unclear [3]. The mechanisms by which cells adapt to a low pH are tissue specific. In neural tissues, cells adapt to changes in the extracellular pH (pHe) by regulating the activities of important pH sensors including acid-sensing ion channel (ASIC) proteins [4].

ASICs are expressed throughout the neurons of the mammalian central and peripheral nervous systems. To date, four ASIC genes (ASIC1–ASIC4) encoding six different subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) have been identified. All ASIC subunits are known to be inhibited by amiloride [5]. Non-steroid anti-inflammatory drugs (NSAIDs) generally used in the treatment of inflammation and pain have an impact on ASIC protein regulation [6]. ASIC1a can be specifically blocked by the tarantula toxin psalmotoxin (PcTX), which was proven to be an effective neuroprotective feature in rodent ischamic brain injury [7,8]. Acidosis is observed in inflammatory conditions such as chronic inflammation, in tumors and after ischemia, and greatly contributes to pain and hyperalgesia [9]. Administration of NSAIDs presumably reduces low-pH-induced pain via ASIC proteins [10].
The aim of this study was to examine the effects of NSAIDs on the regulation and localization of ASICs in nucleus pulposus cells of human intervertebral disk. Our findings showed that ASIC3 was involved in adapting disk cells to their hydrodynamically stressed microenvironment, suggesting an additional role for this ASIC subunit.

**Materials and Methods**

**Patient samples**
Forty out of 294 degeneration patients receiving surgical resection from 2007 to 2010 were randomly selected at the First Affiliated Hospital of Soochow University (Suzhou, China) as the degeneration cases (of the 294 patients, 165 are males and 129 are females, with a male to female ratio of 1.87 : 1, and the patients’ age at surgery ranged from 35 to 81, with an average age of 57.24 years). In addition, three patients with acute spine damage but with no intervertebral disk degeneration were also included as the control cases. The intervertebral disk tissues were obtained from these 43 patients after surgery, and were quickly frozen in liquid nitrogen and maintained at −70°C for further experiments. Patients’ authorized agreement has been acquired for all the process of dealing with their tissues, and the patient studies followed the Patient Care Guidelines in the First Affiliated Hospital of Soochow University.

**Isolation of nucleus pulposus and annulus fibrosus cells**
Nucleus pulposus tissues were surgically isolated from intervertebral disks of all the 43 patients and partially digested by trypsin and collagenase. The isolated nucleus pulposus and annulus fibrosus cells (herein considered as nucleus pulposus cells) were harvested and grown in DMEM/F12 medium (Life Technologies, Grand Island, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml penicillin and 50 μg/ml streptomycin (Life Technologies), and were maintained in a humidified atmosphere containing 5% CO2 at 37°C [11].

The nucleus pulposus cells of the degeneration cases were treated with either amiloride hydrochloride hydrate (1.0 mM; Sigma-Aldrich, St Louis, USA) or NSIAD ibuprofen (100 mM; National Institute for the Control of Pharmaceutical and Biological Products of China, Beijing, China) for 24 h. DMEM/F12 medium only was used as the control. The cells were harvested and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting after treatment.

**Western blot analysis for ASIC proteins**
The levels of the ASIC proteins were analyzed by western blotting as described previously [12]. Primary antibodies against ASIC subunits were used as follows: anti-ASIC1 (E-15, sc-13903, 1 : 1000; Santa Cruz, Santa Cruz, USA); anti-ASIC2 (ASC-012, 1 : 1000; Alomone Labs, Jerusalem, Israel); anti-ASIC3 (ASC-018, 1 : 1500; Alomone Labs); and anti-ASIC4 (N-20, sc-22324, 1 : 1000; Santa Cruz).

**RT-PCR**
RT-PCR was performed as described previously [13]. Primers used were as follows: ASIC1a, 5′-GCCCACTACTTCTCTAT-3″ and 5′-CTTGGTGACGTCGATA-3″; ASIC1b, 5′-CGAGGCGGATCAAAGT-3″ and 5′-CGAAGATCCGAGTCCAT-3″; ASIC3, 5′-ATACAACCGCAGCGGTC-3″ and 5′-TCTTCCTGGGACGAGTGT-3″; β-actin, 5′-TCTTCCTGGGACGAGTGTGTT-3″ and 5′-AAAAGAGGCTGTAACACGCA-3″.

**Electrophysiology**
The electrophysiological recordings were performed with the conventional whole-cell patch recording configuration under voltage-clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a PP83 two-stage puller (Narishige, Tokyo, Japan). The patch electrodes had a resistance of 3–5 MΩ. Whole-cell currents were recorded using Axopatch 1-D amplifier (Axon Instruments, Foster City, USA) in the voltage-clamp mode. Data were filtered at 2 kHz and digitized online using Digidata 1320A DAC units (Axon Instruments). The online acquisition was done using pClamp software pClamp 12.0 (Axon Instruments). The membrane potential was held at −60 mV throughout the experiments. All experiments were carried out at room temperature (22–25°C). Nucleus pulposus cells were washed three times and bathed in extracellular solution containing 150 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM CaCl2, and 2 mM MgCl2, pH 7.4, 320–335 mOsm (Na-rich solution). Patch electrodes contained 150 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 10 mM EGTA, and 4 mg ATP, pH 7.2, 300 mOsm (Na-rich solution). Drugs used in the present experiments were purchased from Sigma. Psalmotoxin-1 (PcTX1) from tarantula Psalmopoeus cambridgei was purchased from Alomone Labs (RTP-100).

Drugs were applied using a rapid application technique termed as the ‘Y-tube’ method throughout the experiment [14]. This system allows a complete exchange of external solution surrounding a cell within 20 ms. After the transient pH drop, cells were incubated with PcTX1 and ibuprofen solution for 10 or 120 s, respectively, before and during new pH changes.

**MTT assay for cell viability**
Cell viability was determined using MTT assay. In brief, at the end of treatment, the cell culture medium of each well was replaced with fresh culture medium (180 μl), followed by the addition of 20 μl of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich), and incubated for 2 h at 37°C. Then the supernatant was removed and 150 μl of dimethyl sulfoxide was added to each well to dissolve the formazan complexes, and then the
absorbance was measured at 570 nm with a Model 680 micro-
plate reader (Bio-Rad, Hercules, USA). Nucleus pulposus
cells receiving vehicle treatment served as the control group.
Cell viability was calculated as a percentage of viable cells in
the experimental group vs. untreated control group.

Statistical analysis
All measurements were made from independent experi-
ments performed in triplicate. Two-group comparisons
were performed using Student’s t-test. Multiple-group comparisons
were performed using one-way analysis of variance and
Fisher’s least-significant difference (equal variances assumed)
or Dunnett’s T3-test (equal variances not assumed). P < 0.05
was considered as statistically different.

Results
ASIC1 and ASIC3 are expressed in cultured nucleus
pulposus cells of the human intervertebral disk
The expression of ASIC proteins was determined as previous-
ly described. First, RT-PCR was performed to verify the
presence of the mRNAs of various ASIC isoforms. As shown
in Fig. 1A, the targeted bands corresponding to ASIC1a,
ASIC1b, and ASIC3 were detected in cultured nucleus pulpo-
sus cells of the human intervertebral disk. Furthermore,
western blot analyses of the nucleus pulposus cells of the
human indicated that the cells express ASIC1 and ASIC3 pro-
teins (Fig. 1B,C).

ASIC1 and ASIC3 expression are significantly increased
by chronic degeneration
Chronic degeneration significantly increases the ASIC1 and
ASIC3 expression in nucleus pulposus cells of the human
intervertebral disk (Fig. 1B,C). These data consistently
demonstrated the existence of isoforms ASIC1a, ASIC1b,
and ASIC3 in cultured nucleus pulposus cells of the human
intervertebral disk. Other ASIC isoforms could not be
detected in the disk tissues. Pictures of nucleus pulposus
cells of the human intervertebral disk were shown under an
optical microscope in four different magnifications, and
nucleus pulposus cells were defined by Type II collagen and
aggrecan cell marker (Fig. 1D).

Figure 1. Detection of ASICs transcripts and expression in nucleus pulposus cells of human intervertebral disc cells
(A) Expression of mRNA of ASIC1a, ASIC1b, and ASIC3 subunits in nucleus pulposus cells of the human intervertebral disc cells was determined by a non-quantitative RT-PCR
analysis. Nucleus pulposus cells from normal and degenerated tissue were investigated for ASIC1 (B) and ASIC3 (C) expression by western blot. Equal
loading is proven by actin expression. Representative densitometry analysis of ASIC expression calculated against untreated controls set. Bars represent
mean ± SE, n = 5–9 per group. **P < 0.05 (degeneration group vs. control group). (D) Nucleus pulposus cells of the human intervertebral disc were
proven as nucleus pulposus cells under the optical and the fluorescence microscopes. (a–d) Optical microscope images of nucleus pulposus cells with
magnification of ×100 (scale bar 120 μm), ×200 (scale bar scale bar 60 μm), ×400 (scale bar 30 μm), and ×600 (scale bar 20 μm) size are as indicated by
scale bars. Immunofluorescence images of NP cells stained for Type II collagen (e) and aggrecan (f) are shown, sizes are indicated by scale bar correlating
with ×600 magnification.
NSAID ibuprofen attenuates the upregulation of ASIC expression in nucleus pulposus cells of chronic degeneration

Further studies were performed to test the effect of NSAID ibuprofen on the expression of ASICs, in addition to the ASIC current. Compared with the degeneration group, amiloride (1 mM) fully reduced ASIC upregulation in nucleus pulposus cells of the human intervertebral disk (Fig. 2A,B). Similarly, treatment with 100 μM ibuprofen also remarkably alleviated the increase of ASIC expression in nucleus pulposus cells of chronic degeneration (Fig. 2C,D). These data indicated that ibuprofen inhibited both the expression and current of ASIC1 and ASIC3 induced by acidic stress.

Acid activates the inward current in nucleus pulposus cells of the human intervertebral disk

The expression and function of ASICs in nucleus pulposus cells of the human intervertebral disk cells were investigated by electrophysiological recording. It was observed that a rapid drop in pH, from 7.4 to 6.0 evoked a transient in nucleus pulposus cells from the control cases, rapidly inactivating the inward current (Fig. 3A). The amplitude of ASIC current in nucleus pulposus cells of the human intervertebral disk decreased slightly following the formation of whole-cell configuration. In most nucleus pulposus cells of the human intervertebral disk, the threshold pH to elicit the inward current was \(~7.4\) and the maximum response appeared at 5.0–6.0.

![Figure 2. Amiloride and ibuprofen treatment decreased ASIC expression](image-url)

Nucleus pulposus cells from normal and degenerated tissue were treated with amiloride (1 mM) or ibuprofen (100 μM) for 24 h and ASIC1 (A, C) and ASIC3 (B, D) expression was determined by western blotting. Increased expression of ASIC1 and ASIC3 was reduced by both amiloride and ibuprofen treatment. Representative densitometry analysis of ASIC expression calculated against untreated controls set. Bars represent mean \(\pm\) SE, \(n = 5–9\) per group. **\(P < 0.05\) (degeneration group vs. control group); ##\(P < 0.05\) (amiloride or ibuprofen-treated + degeneration group vs. degeneration group); &&\(P < 0.05\) (amiloride or ibuprofen-treated + degeneration group vs. control group).
NSAID ibuprofen blocks ASIC currents in nucleus pulposus cells of the human intervertebral disk

Amiloride, a well-known blocker of ASICs, was used as a positive control to test the action of ibuprofen on proton-gated currents in nucleus pulposus cells of the human intervertebral disk. Inward currents were activated by a pH drop from 7.4 to 6.0 at a holding potential of −60 mV. After recording of two to three consecutive traces that had similar amplitude, cumulative concentrations of amiloride were added to both pH 7.4 and pH 6.0 solutions. Enough time was allowed for the effect of each concentration of amiloride to be stabilized. When the final concentration of amiloride was stabilized, the drug was washed out. These proton-gated currents in nucleus pulposus cells of the human intervertebral disk were reversibly inhibited by amiloride in a dose-dependent manner (Fig. 3C). In addition, proton-activated current was also shown to be dose dependently blocked by PcTX1, a specific inhibitor of ASIC1a (Fig. 3B). Likewise, ibuprofen was found to block the ASIC current in nucleus pulposus cells of the human intervertebral disk in a dose-dependent manner, as shown in Fig. 3D. These data showed that the ASIC currents in nucleus pulposus cells of the human intervertebral disk could be inhibited by ibuprofen, implying its protective effects on cell damage by inhibiting ASICs.
NSAID ibuprofen protects against degeneration-induced cell death in nucleus pulposus cells of the human intervertebral disk

Furthermore, the potential effects of ibuprofen and amiloride on degeneration-induced cytotoxicity in nucleus pulposus cells of the human intervertebral disk were assessed by measuring the cell viability with MTT assay. Exposure of nucleus pulposus cells of the human intervertebral disk to degeneration induced a significant decrease of cell viability. Co-treatment with amiloride (1 mM) was found to partially rescue the cells from degeneration-induced injury (Fig. 4A). Co-treatment with ibuprofen produced similar protective effects (Fig. 4B).

Discussion

In this study, we confirmed that the nucleus pulposus and inner annulus fibrosus cells express ASIC proteins. This is particularly interesting as disk cells are of notochordal in origin [15], and do not contain either sensory or motor neurons under physiological conditions [16]. Recently, Cuesta et al. [17] showed the presence and localization of different ASICs in the human healthy intervertebral disk, and an increased expression of ASICs in the degenerated intervertebral disk, indicating that ASICs may be involved in intervertebral disk degeneration. In this study, we investigated two ASIC subunits (ASIC1 and ASIC3) in nucleus pulposus cells of the human intervertebral disk, which were reported to be relevant for the function of connective tissues including bone and cartilage [18–20]. Uchiyama et al. [21] confirmed the presence of ASIC3 subunit in nucleus cells of the intervertebral disk. In our study, we further showed the expression of ASIC1 in nucleus cells of the intervertebral disk.

We assume that the functional activity of these channel proteins is linked to the glycolytic status of the investigated tissues. In the disk, the limited vascularity is expected to result in an accumulation of glycolytic byproducts, which might contribute to a decrease in the disk pH and an increase in the osmolarity of the extracellular matrix [22,23]. pH-dependent Na⁺ flux through the ASIC proteins might elevate the cation concentration and thereby promote restoration of the osmotic status in the tissue. Thus, the ASIC proteins in the disk tissue could function as ‘osmosensors’ countering the hydrodynamical stress and thereby supporting cell survival in that microenvironmental niche, rather than only serving as neurosensory molecules [24,25].

NSAIDs are commonly used to treat pain and inflammation associated with osteoarthritis [26–28]. However, several studies have reported conflicting effects (favorable or detrimental) of NSAIDs on osteoarthritis progression and articular cartilage [29,30]. We consider ASICs as new potential targets for the treatment of pain and inflammation, because ASICs appear to be important for cell survival [31,32]. Here, we investigated the potential protective effects of NSAIDs on the dorsal disk prolapse. Our results suggest that ASICs are involved in the development of dorsal disk prolapse induced by deep tissue injury. This would attribute to an additional role for ASICs as a pH sensor in the development of dorsal disk prolapse hyperalgesia after aging. Particularly, ASIC1α was assumed to mediate dorsal disk prolapse after a moderate extracellular pH decrease. Since homomeric ASIC1b and ASIC4 were not involved in membrane potential [8], the currents we detected here were presumably from ASIC1α and ASIC3 together (homomeric or heteromeric). To further delineate the specific impact of ASIC1α and ASIC3, we would need to use specific inhibitors of ASIC3 (APETx2), which is beyond our current aim, but interesting for the follow-up studies. NSAIDs also prevented the large inflammation or aging-induced increase of ASICs expression [33,34].

Many painful inflammatory and ischemic conditions such as rheumatoid arthritis, cardiac ischemia, and exhausted...
skeletal muscles are accompanied by local tissue acidosis. In such acidotic situation, extracellular protons provoke the pain by opening cation channels in nociceptors [33,35]. Navone et al. [36] acquired nucleus pulposus of intervertebral disks isolated from 14 human pathological disks which were cultured with mesenchymal cells and differentiated to neural cells. In addition to Navone et al., we were able to include a small number of disk tissue samples from patients with acute spine damage. This further confirmed that chronic disk injury is associated with an upregulation of ASIC1. The nucleus pulposus tissues could not be obtained from healthy humans because of ethics, so three disk tissue samples from patients with acute spine damage were considered as control cases with no degeneration. Compared with 40 patients with disk degeneration, the number in the control group is very small. Thus, there was limitation that the comparison with the degeneration group is statistically weakened. In these cells, the expression of TrkB, the BDNF receptor, was increased and was co-expressed with ASIC3. Under the same conditions, neuroinflammatory markers were over-expressed. Navone et al. [36] confirmed that stem cells with an intervertebral disk degeneration acquired neurogenic phenotype, resulting in the induction of markers related to inflammatory condition. To explore the functional role of ASIC proteins, nucleus pulposus cells were treated with amiloride, a known ASIC inhibitor, to inhibit ASIC-mediated effects on cells. Our data here showed that amiloride, PcTX1, and ibuprofen decreased ASIC protein expression and thereby exerted neuroprotective features. In conclusion, the present study revealed that bone cell function was modulated by environmental pH under physiological (e.g. bone resorption) and pathological (e.g. metabolic acidosis) conditions. Recently ASIC protein family was found also to function as a pH sensor in nucleus pulposus cells of the human intervertebral disk, this further enhances our understanding of human intervertebral disk in health and disease.

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