Involvement of acid-sensing ion channel 1a in matrix metabolism of endplate chondrocytes under extracellular acidic conditions through NF- κ B transcriptional activity

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Abstract Acidic conditions are present in degenerated intervertebral discs and are believed to be responsible for matrix breakdown. Acid-sensing ion channel 1a (ASIC1a) is expressed in endplate chondrocytes, and its activation is associated with endplate chondrocyte apoptosis. However, the precise role of ASIC1a in regulating the matrix metabolic activity of endplate chondrocytes in response to extracellular acid remains poorly understood. Aggrecan (ACAN), type II collagen (Col2a1), and matrix metalloproteinase (MMP) expressions were determined using reverse transcription (RT)-PCR and Western blot. ASIC1a was knocked down by transfecting endplate chondrocytes with ASIC1a siRNA. MMP activity and NF- κ B transcriptional activity were measured. NF- κ B transcriptional activity was assessed by examining cytosolic phosphorylated I κ B α and nuclear phosphorylated p65 levels. Extracellular acidic solution

(pH 6.0) resulted in a decrease in ACAN and Col2a1 expressions and an increase in MMP-1, MMP-9, and MMP-13 expressions, as well as in MMP activity; while ASIC1a siRNA blocked these effects. In addition, acid-induced increase in cytosolic levels of phosphorylated I κ B α and nuclear levels of phosphorylated p65 in endplate chondrocytes were inhibited by ASIC1a siRNA. ASIC1a is involved in matrix metabolism of endplate chondrocytes under extracellular acidic conditions via NF- κ B transcriptional activity.

Keywords Acid-sensing ion channel 1a · Extracellular pH · Metalloproteinases · Endplate chondrocytes

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Introduction

Intervertebral disc degeneration (IVDD) is characterized by a decrease in cellularity in intervertebral disc (IVD), leading to a loss of extracellular matrix (ECM) structure, altered biomechanical loading, and clinical presence of pain (Maidhof et al. 2012). The IVD is the largest avascular tissue in the body that obtains all essential nutrients such as oxygen and glucose through the cartilage endplate (CEP) (Grunhagen et al. 2006; Malandrino et al. 2014). The CEP connects the superior and inferior surfaces of the IVD to the cortical bone of the vertebral bodies and is an unusual tissue that contains only sparsely populated chondrocytes (Yuan et al. 2014). Chondrocytes play a vital role in the development and maintenance of ECM and are regulated by a great variety of stimuli in both normal and pathophysiologic states. For example, during diseased states, chondrocytes are stimulated in response to abnormal stimuli such as overloading, hypoxia, and acidosis, resulting in an uncontrolled ECM turnover (Sakai and Grad 2014).

Several studies have shown that CEP degeneration causes lactic acid build-up, resulting in a decrease in pH in the IVD

(Bibby and Urban 2004; Ishihara and Urban 1999; Razaq et al. 2003). Normal pH is necessary to maintain normal matrix turnover in the CEP, whereas excessive low pH values triggers the destruction of the ECM structure. Cartilage ECM contains a high percentage of collagen fibers and proteoglycans, which can be directly cleaved by ECM-degrading enzymes such as matrix metalloproteinases (MMPs) (Su et al. 2014). It has been shown that extracellular acid induces the production of MMPs and inhibits matrix synthesis in disc cells and chondrocytes (Razaq et al. 2003). However, acid-sensing mechanisms and mechanisms by which extracellular acid regulates ECM production and degradation in endplate chondrocytes have not been fully elucidated.

In the recent years, great progress has been made towards understanding cellular sensory mechanisms by which cells detect changes in extracellular pH. Several classes of proteins, including transient receptor potential V1 (TRPV1), acid-sensing ion channels (ASICs), and proton-sensing G-protein-coupled receptors (GPCRs) are major extracellular receptors that respond to acidosis (Cao et al. 2013; Justus et al. 2013; Xiong et al. 2004). ASICs belong to a family of Na^+ -selective and Ca^{2+} -permeant ligand-gated cation channels, which are activated by extracellular protons (Waldmann et al. 1997). Acid-sensing ion channel 1a (ASIC1a) is a member of ASICs, which is permeable to Ca^{2+} and responsible for acidosis-mediated cell injury (Yuan et al. 2010). Our previous study suggested that ASIC1a mediated an increase in intracellular Ca^{2+} concentration and activated Ca^{2+} -dependent proteases, which eventually led to endplate chondrocyte apoptosis in IVDs (Li et al. 2014). However, the precise role of ASIC1a in regulating the metabolic activity of endplate chondrocytes in response to extracellular acid remains unclear.

In this study, we examined the effects of extracellular acid on the production and degradation of ECM components in the CEP and investigated the role of ASIC1a in mediating these effects. We hypothesized that ASIC1a activation by extracellular acid plays a central role in CEP matrix maintenance.

Materials and methods

Culture and treatment of rat endplate chondrocytes

Chondrocytes were isolated from the endplate cartilage of 4-week-old Sprague–Dawley rats, as described previously (Li et al. 2014). Briefly, cartilage of L1–L5 endplates were carefully removed from the intervertebral body and minced into small pieces (approximately 1 mm^3). Cartilage tissues were transferred into a 50-mL tube and digested with a 0.25 % trypsin solution (Hyclone, Salt Lake City, UT, USA) at 37°C for 15 min, followed by 0.02 % collagenase (Sigma, St. Louis, MO, USA) at 37°C for 24 h. Then, digested tissues were passed through a 100- μm cell strainer (BD Biosciences,

Bedford, MA, USA). Chondrocytes were isolated from the rats with the yield of $1.96 \pm 0.08 \times 10^6$ cells for each endplate cartilage. The cells were washed three times with phosphate-buffered saline (PBS), plated into 25-mL tissue culture flasks (Corning Inc., Corning, NY, USA) at a density of 2×10^4 cells/ cm^2 and cultured at 37°C in 5 % CO_2 . Primary chondrocytes were cultured for 1 week in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco) and used for the following experiments. Identification of cultured chondrocytes was confirmed by toluidine blue staining and immunohistochemistry analysis.

To study the effects of acidic stimulation on ASIC1a, extracellular pH in the extracellular media was adjusted by adding an appropriate amount of HCl (Li et al. 2014). The pH value was measured with a pH meter (Radiometer pHM82, Copenhagen, Denmark). Blockers of voltage-gated Ca^{2+} channels (5 μM nimodipine and 3 μM ω -conotoxin MVIIC), glutamate receptors (10 μM MK-801), TRPV1 (2 μM capsazepine), and OGR1 (100 μM , CuCl_2) were added to the extracellular solutions to inhibit these channels. The cells were first treated with extracellular solutions at pH 6.0 in different time points; then, the cells were washed three times with normal extracellular solutions (140 mM NaCl, 5.4 mM KCl, 25 mM HEPES, 20 mM glucose, 1.3 mM CaCl_2 , 1.0 mM MgCl_2 , and 0.0005 mM tetrodotoxin; pH 7.4).

siRNA transfection

siRNA transfection was performed as described in our previous study (Li et al. 2014).

Real-time RT-PCR analysis

The total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. The total RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Fermentas China Co., Ltd., Shanghai, China). Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and GeneAmp 5700 Sequence Detection System. Primers used in the study are shown in Table 1. Normalized cycle threshold (Ct) values were used for comparison. Each sample was analyzed in triplicates to ensure accuracy.

Western blot analysis

Chondrocytes were homogenized on ice in lysis buffer, containing protease inhibitor mixtures (Roche Applied Science, Indianapolis, IN). Nuclear and cytosolic proteins were isolated using a Nuclear/Cytosol Fractionation Kit (BioVision, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The protein concentrations were determined using a BCA-200 protein assay kit (Pierce, Rockford, IL,

Table 1 Primers used in qRT-PCR

Gene	Sense primer	Antisense primer
Col2a1	GAGTGGAAAGACGGAGACTACTG	CTCCATGTTGCAGAAGACTTTCA
ACAN	TTGTGACTCTGCGGGTCATC	GTCCCTAGGAGGGCCTTCAG
MMP-1	GACGTGGACCGACAACAGTGA	GGGGAACATTAGTGCTCCTACATC
MMP-3	CTCTTCCATCCAACATGGATAGCTG	ACTCCAACGTGAAGATCCGCTGA
MMP-9	AGCGCCAGCCGACTTATGTGG	ACACCACAGCGCGGTGAACG
MMP-13	CCCTGGAGCCCTGATGTTT	CTCTGGTGTTTGGGGTGCT
GAPDH	GGCACAGTAAGGCTGAGAAT	TCTCGCTCCTGGAAGATGGT

USA). The proteins were separated by 12 % SDS-PAGE and transferred onto a polyvinyl difluoride (PVDF) membrane (Pierce). Then, the membrane was blocked with 5 % skimmed milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris, 150 mM NaCl, and 0.05 % Tween 20; pH 8.3). The membrane was incubated with primary antibodies against aggrecan (ACAN; lot # SAB4500662, Sigma, USA), type II collagen (Col2a1; lot # sc-28887, Santa Cruz Biotech, USA), MMP-1, MMP-9, and MMP-13 (lot # sc-30069, lot # sc-10737, lot # sc-30073, Santa Cruz Biotech, USA), or phosphorylated NF- κ B (lot # 3039S, Cell Signaling Technology, USA) at 4 °C overnight. The membranes were washed in TBST and incubated with secondary antibodies for 1 h at

room temperature. Protein band intensities were quantified by densitometry (Gel Logic 2200; Rochester, NY, USA).

MMP activity assay

Generic MMP activity of endplate chondrocytes was assayed using the SensoLyte 520 generic MMP activity kit (AnaSpec) according to the manufacturer's instructions. Isolated protein samples were incubated with 4-aminophenylmercuric acetate for 24 h to activate pro-MMPs. Then, MMP substrates were incubated with the activated proteins for 30 min at room temperature, and fluorescence signals were determined.

Fig. 1 ASIC1a is involved in extracellular acid-induced inhibition of ACAN and Col2a1 expressions in endplate chondrocytes. **a, b** qRT-PCR results showing ACAN (**a**) and Col2a1 (**b**) relative mRNA expressions in endplate chondrocytes treated with extracellular acid solution (pH 6.0) for 0–24 h. $n=12$; $**P<0.01$ vs. control (pH 7.4); $##P<0.01$ vs. pH 6.0. **c** Representative Western blot analysis of ACAN and Col2a1 expressions in endplate chondrocytes treated with extracellular acid solution (pH 6.0) for 24 h. **d** Quantification of ACAN and Col2a1 protein expression levels. $**P<0.01$ vs. control; $##P<0.01$ vs. pH 6.0

Luciferase reporter assay

NF- κ B transcriptional activity was measured using a luciferase reporter assay. The cells were transfected with 1 μ g of pGL4.32 [luc2P/NF- κ B-RE/Hygro] vector and 1 μ g of pGL4.44 [luc2P/AP1 RE/Hygro] vector (Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen). To detect luciferase activity, chondrocytes were lysed with 1 $\times$ passive lysis buffer (Promega, USA). Then, cell lysates were mixed with luciferase assay substrates. Luciferase activity was measured using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, USA).

Statistical analysis

Data are expressed as mean $\pm$ standard error. Student's *t* test was used to compare differences between the two groups. One-way analysis of variance (ANOVA) was used to compare differences among three or more groups, followed by a least significant difference (LSD) post hoc test. *P* values <0.05 were considered significant.

Results

Involvement of ASIC1a in acid-induced inhibition of ACAN and Col2a1 synthesis in endplate chondrocytes

The cultured cells were identified to be endplate chondrocytes by toluidine blue staining of glycosaminoglycan (GAG)

(Figure S 1) and immunohistochemical staining of type II collagen (Figure S 1). The cells were cultured for 1 week and used for the experiment. Reverse transcription (RT)-PCR results showed that the cells were at an intermediate stage of chondrogenic differentiation (Figure S1). To explore whether ASIC1a affects acid-induced matrix degradation, siRNAs targeting ASIC1a were transfected into endplate chondrocytes, followed by acidic pH (pH 6.0) treatment for 24 h. Acidic conditions induced ACAN and Col2a1 mRNA expressions to decrease in a time-dependent manner (Fig. 1a, b). ASIC1a siRNA attenuated the acid-induced downregulation of ACAN and Col2a1 expressions in endplate chondrocytes. Negative control siRNA (NC-RNAi) had minimal effects on the acid-induced decrease in ACAN or Col2a1 mRNA expressions. Consistent with RT-PCR results, Western blot analysis showed that ASIC1a-specific siRNA, except for NC-RNAi, reduced the acid-induced decrease in ACAN and Col2a1 protein expressions (Fig. 1c, d). Thus, our data suggested that ASIC1a activation was involved in the acid-induced catabolic destruction of endplate cartilage.

ASIC1a mediates acid-induced increase in MMP expressions in endplate chondrocytes

Acidic extracellular environments have been shown to induce matrix-degrading enzyme production in chondrocytes (Razaq et al. 2003). MMP-1 and MMP-9 mRNA expressions in endplate chondrocytes significantly increased after acidic treatment (pH 6.0) for 6–24 h (Fig. 2a, b), and MMP-3 and MMP-13 mRNA expressions were significantly upregulated after acidic treatment for 12–24 h (Fig. 2c, d). Acid-induced increase in MMP-1, MMP-9, and MMP-13 mRNA

Fig. 2 ASIC1a is involved in extracellular acid-induced increase in MMP expression in endplate chondrocytes. **a, b, c** and **d** qRT-PCR results showing MMP-1 (**a**), MMP-9 (**b**), MMP-13 (**c**), and MMP-3 (**d**) relative mRNA expressions in endplate chondrocytes treated with extracellular acid solution (pH 6.0) for 0–24 h. *n*=12; ***P*<0.01 vs. control (pH 7.4); ##*P*<0.01 vs. pH 6.0

expressions in endplate chondrocytes was attenuated by ASIC1a-siRNA transfection, but not in the NC-RNAi (Fig. 2a–c). In contrast, acid-induced increase in MMP-3 mRNA expression was not inhibited by ASIC1a-siRNAi (Fig. 2d). These data suggested that ASIC1a mediated acid-induced transcriptional activation of MMPs in endplate chondrocytes.

ASIC1a mediates acid-induced increase in MMP protein levels and activities in endplate chondrocytes

To determine whether ASIC1a mediates the acid-induced up-regulation of MMP protein levels, MMP-1, MMP-9, and MMP-13 protein expressions in endplate chondrocytes were assessed by Western blot. MMP-1 and MMP-13 protein expressions were significantly higher in endplate chondrocytes after 6–24 h of acidic treatment (Fig. 3a, b), and MMP-9 expression significantly increased after 12–24 h of acid

treatment (Fig. 3a, b). Acid-induced MMP-1, MMP-9, and MMP-13 upregulation was inhibited by ASIC1a-siRNA but not by NC-RNAi (Fig. 3a, b).

The time course of acid-induced increase in MMP activities in endplate chondrocytes coincided with the acid-induced increase in protein expression (Fig. 3c). ASIC1a-siRNA treatment reduced the acid-induced increase in MMP activity, while negative NC-RNAi had minimal effects.

ASIC1a regulates acid-induced MMP production via NF- κ B phosphorylation

NF- κ B is one of the most important transcription factors that regulate MMP and aggrecanase expressions in many cell types (Vincenti and Brinckerhoff 2002). Inactive NF- κ B consists of a heterotrimer, which comprises of p50 and p65 sub-units, and the I κ B α protein. I κ B α phosphorylation releases the p50–p65 heterodimer and translocates to the nucleus

Fig. 3 ASIC1a mediates acid-induced increase in MMP expression and activity in endplate chondrocytes. **a** Representative Western blot analysis of MMP-1, MMP-9, and MMP-13 protein expressions in endplate chondrocytes treated with extracellular acid solution (pH 6.0) for 0–24 h. **b** Quantification of MMP-1, MMP-9, and MMP-13 relative

protein expression levels. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. pH 6.0. **c** MMP activity in culture medium of endplate chondrocytes treated with extracellular acidic solution (pH 6.0) for 1–24 h. MMP activity was measured using a generic MMP assay kit. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. pH 6.0

where it induces a specific gene expression. Since our results indicated that ASIC1a acts as a triggering event in the acid induction of MMP protein expression and activity in endplate chondrocytes, we investigated whether acid induced MMP production in endplate chondrocytes via NF- κ B transcriptional activity. A NF- κ B inhibitor was used to inhibit NF- κ B transcriptional activity to investigate whether NF- κ B mediated the acid-induced increase in MMP-1, MMP-9, and MMP-13 expressions, as well as in pyrrolidine dithiocarbamate (PDTC). PDTC (100 μ M) significantly reduced the acid-induced increase in MMP-1, MMP-9, and MMP-13 mRNA expressions (Fig. 4a), suggesting that acid-induced increase in MMP expression was dependent on NF- κ B transcriptional activity.

To directly examine the involvement of ASIC1a in the acid-induced activation of NF- κ B in chondrocytes, NF- κ B transcriptional activity levels were tested using a NF- κ B luciferase reporter gene assay. NF- κ B activity began to rise after 6 h of acidic treatment and reached its peak at 12 h after acidic treatment. Acid-induced increase in NF- κ B activity was blocked by ASIC1a-siRNA, but not by NC-RNAi, suggesting that ASIC1a was required for the acid-induced activation of NF- κ B in endplate chondrocytes. In addition, Western blot analysis showed that extracellular acid significantly increased nuclear NF- κ B p65 phosphorylation (Fig. 4c), and ASIC1a-siRNA treatment attenuated the acid-induced increase in nuclear NF- κ B p65 phosphorylation (Fig. 4c). Moreover,

extracellular acid increased the cytosolic levels of phosphorylated I κ B α , an indicator of NF- κ B transcriptional activity (Fig. 4c), while ASIC1a-siRNA treatment blocked acid-induced increase in cytosolic levels of phosphorylated I κ B α (Fig. 4c). Our results suggested that ASIC1a contributes to acid-induced MMP production by NF- κ B transcriptional activity.

Discussion

Our results confirm our hypothesis that ASIC1a plays a critical role in regulating matrix metabolism in endplate chondrocytes in response to extracellular acid. This study revealed that ASIC1a knockdown in endplate chondrocytes attenuated the acid-induced inhibition of ACAN and Col2a1 synthesis, which may be associated with the increased MMP-1, MMP-9, and MMP-13 expressions, as well as in MMP activity. Furthermore, we found that ASIC1a was involved in the acid-induced activation of NF- κ B signaling pathways in endplate chondrocytes.

In healthy IVD endplate cartilages, endplate chondrocytes survive in unusual physicochemical, mechanical, and biological environments within the ECM (Urban et al. 2004). Endplate chondrocytes are responsible for the maintenance and turnover of cartilage-specific ECM molecules including Col2a1 and ACAN, endowing endplate cartilages with its

Fig. 4 ASIC1a is involved in the acid-induced production of MMPs by regulating NF- κ B phosphorylation. **a** RT-PCR results showing MMP-1, MMP-9, and MMP-13 relative mRNA expressions in control cells, cells treated with acid solution (pH 6.0), and cells treated with acidic solution

(pH 6.0) and PDTC (100 μ M). ** P <0.01 vs. control; ## P <0.01 vs. pH 6.0. **c** Western blot results showing NF- κ B, I κ B, and p65 expression levels. ** P <0.01 vs. pH 6.0 group at 24 h

load-bearing properties, while allowing nutrient exchange (Herrero et al. 2014; Jackson et al. 2011). Endplate ECM plays a major role in the initiation and development of IVD degeneration. The decrease in extracellular matrix pH has been found to regulate protein and proteoglycan synthesis in intervertebral disc explants and other types of cartilages (Bibby et al. 2005; Bibby and Urban 2004; Wang et al. 2011). Interestingly, we found that ASIC1a knockdown by siRNA suppressed the acid-induced downregulation of ACAN and Col2a1 expressions. Thus, ASIC1a possibly mediates endplate ECM degradation induced by extracellular acid in IVDs.

Among all catabolic factors involved in IVD endplate ECM degradation, MMPs play a crucial role in collagen and proteoglycan degradation (Razaq et al. 2003; Roberts et al. 2000). It has been shown that the decrease in extracellular pH induced a steep increase in total MMP production in disc cells and in articular chondrocytes (Razaq et al. 2003). Consistent with the previous study, we found that extracellular acid increased MMP-1, MMP-3, MMP-9, and MMP-13 mRNA expressions in endplate chondrocytes of rat IVDs. ASIC1a knockdown inhibited acid-induced increase in MMP-1, MMP-9, and MMP-13 mRNA and protein expressions in endplate chondrocytes, except in MMP-3. Moreover, ASIC1a knockdown suppressed the acid-induced elevation in MMP activity in endplate chondrocytes. Our data suggests that ASIC1a activation in endplate chondrocytes may result in an increase in endplate ECM degradation in the IVD.

NF- κ B is a transcription factor in almost all types of animal cells and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens (Ledoux and Perkins 2014; Luqman and Pezzuto 2010). Activation of the NF- κ B signaling pathway has been shown to be involved in regulating MMP transcription in chondrocytes (Ma et al. 2013). We found that the specific NF- κ B inhibitor PDTC (100 μ M) significantly reduced the acid-induced increase in MMP expression in endplate chondrocyte, suggesting that NF- κ B transcriptional activity played an important role in acid-induced MMP production. In addition, we also found that ASIC1a siRNA-treated endplate chondrocytes reduced NF- κ B phosphorylation levels. Thus, our findings suggest that NF- κ B is involved in regulating endplate chondrocyte ECM metabolism by extracellular acid.

In summary, our results indicate that ASIC1a activation by extracellular acid induces ECM metabolism in rat endplate chondrocytes by increasing MMP expression and activity through NF- κ B transcriptional activity. Thus, ASIC1a may provide a molecular basis for sensing acid stress in IVD pathogenesis, and targeting ASIC1a may be a novel therapeutic strategy for IVDD treatment.

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Conflict of interest The authors declare no conflicts of interest.

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