Original Paper

Transient Receptor Potential Vanilloid 5 Mediates Ca^{2+} Influx and Inhibits Chondrocyte Autophagy in a Rat Osteoarthritis Model

Yingliang Wei Yanfang Wang Yutong Wang Lunhao Bai

Department of Orthopedic Surgery, ShengJing Hospital, China Medical University, ShenYang, People's Republic of China

Key WordsTRPV5 • Ca^{2+} • Chondrocyte • Autophagy • Osteoarthritis**Abstract**

Background: Autophagy, a self-protective mechanism of chondrocytes, has become a promising target to impede the progress of osteoarthritis (OA). Autophagy is regulated by cytosolic Ca^{2+} activity and may thus be modified by the Ca^{2+} permeable transient receptor potential channel vanilloid 5 (TRPV5). Therefore, we investigated the potential role of TRPV5 in mediating Ca^{2+} influx and in inhibiting chondrocyte autophagy in a rat OA model. **Methods:** The rat OA model was assessed by macroscopic and histological analyses. light chain 3B (LC3B) immunolocalization was detected by immunohistochemistry. TRPV5, LC3B and calmodulin in OA articular cartilage were assessed by real time polymerase chain reaction (RT-PCR) and western blotting. TRPV5 small interfering RNA (TRPV5 siRNA) were transfected into rat primary chondrocyte then the calmodulin and LC3B was detected by immunofluorescence. The functionality of the TRPV5 was assessed by Ca^{2+} influx. Western blot was used to measure autophagy-related proteins. **Results:** We constructed a monosodium iodoacetate (MIA)-induced rat OA model and found that ruthenium red (TRPV5 inhibitor) slowed the progression of joint destruction. We found that the TRPV5 and calmodulin were up-regulated but LC3B was down-regulated in articular cartilage following prolonged progression of OA. Furthermore, the up-regulated TRPV5 channel caused an increase in the Ca^{2+} influx in chondrocytes. The up-regulation of TRPV5 stimulated Ca^{2+} influx, which inhibited autophagy by increasing the production of calmodulin, phosphorylation of calmodulin dependent protein kinases II (p-CAMK II), phosphorylation of Beclin1 (p-Beclin1), and protein of B-cell lymphoma-2 (Bcl-2), and attenuating ratio of LC3- II / LC3-. **Conclusion:** Up-regulated TRPV5 as an initiating factor inhibited chondrocyte autophagy via the mediation of Ca^{2+} influx.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Osteoarthritis (OA) can cause pain, stiffness, swelling, and loss of function in the joints, which greatly affects quality of life in the elderly population [1]. Pathologically, OA is characterized by the progressive degeneration of articular cartilage, synovial inflammation, and subchondral bone remodeling [2]. The process of articular cartilage degeneration mainly involves cartilage chondrocyte breakdown during homeostasis. Autophagy, a self-protective mechanism in chondrocytes, has become a promising target to improve the viability and function of chondrocytes, thereby impeding the progress of OA [3, 4]. *In vitro*, autophagic inhibition stimulates apoptosis and an increase of matrix metalloproteinases in chondrocytes [5, 6]. Specifically, the relationship between compromised autophagy and the severity and progression of OA has been confirmed in many studies [7, 8]. Thus, in the progression of cartilage degeneration, finding a cause for the inhibition of chondrocyte autophagy during OA may become a potential strategy against this disease.

Ca^{2+} is a major intracellular second messenger and a key regulator of cell survival. The breakdown of Ca^{2+} homeostasis due to its sustained elevation in the cytoplasm was able to inhibit autophagy [9, 10]. The role of Ca^{2+} influx through Ca^{2+} selective channels, such as the transient receptor potential channel vanilloid (TRPV), has recently been studied more extensively [11, 12]. In addition, Matsumoto et al. showed that cytosolic calcium regulates autophagy-related gene expression in autophagy-deficient mice [13]. The increase in intracellular calcium levels can result in the activation of calcium sensing proteins, such as calmodulin, which can combine with a target protein to form a Ca^{2+} /calmodulin complex [14]. The Ca^{2+} /calmodulin complex can act as a kinase that participates in calmodulin-dependent protein kinase 2 (CAMK II) activation [15]. Activated CAMK II can act as a kinase, activating downstream autophagy-related proteins to inhibit autophagy and eventually lead to the reduction of autophagosome formation.

TRPV5 is a member of the TRPV subfamily and functions as a facilitative transporter of calcium [16]. Recent knowledge of TRPV5 channel physiology is still relatively limited. What is known is that transmembrane Ca^{2+} transport is necessary for normal physiology. Once this homeostasis is broken, cells may become pathologically changed, and even die. TRPV5 is particularly important in the regulation of Ca^{2+} influx to maintain Ca^{2+} homeostasis. However, there is little evidence suggesting that TRPV5 may be expressed in articular chondrocytes. Even less information is available on the relationship between TRPV5 and OA, a chronic articular disease.

The purpose of this study was to evaluate expression levels of TRPV5 in normal and OA articular cartilage. The role and potential regulatory mechanisms involved in TRPV5-mediated Ca^{2+} influx and inhibition of chondrocytes autophagy in a monosodium iodoacetate (MIA)-induced rat OA model will also be examined. Such studies may yield a potential new therapeutic target for OA treatment.

Materials and Methods

MIA-induced rat OA model

Male Sprague-Dawley rats (220 to 230 g in weight) were housed in groups of five per cage under standard laboratory conditions with constant room temperature (22°C) and humidity (45% to 50%). MIA (Sigma-Aldrich, St Louis, MI, USA) was dissolved in sterile saline. Rats were randomly divided into groups as described below (Fig. 1A). Rats were given an intra-articular injection of MIA and ruthenium red through the infra-patella ligament of a random knee at a dose of 1 mg in 50 μL sterile saline; control (normal) animals were given an intra-articular injection of an equal volume of sterile saline. All experiments were carried out in accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals (publication number 85-23, revised 1985) and the Care and Use of Animals in Medical Research Center, and were approved by China Medical University.

Macroscopic analysis

Joint space was monitored using digital X-rays (MX-20, Faxitron X-Ray Corp., Wheeling, IL, USA). X-rays were graded as follows: 0 = normal appearance; 1 = slight narrowing of the joint space; 2 = narrowing of the joint space but with no osteophytes; 3 = severe narrowing of the joint space with some osteophytes; and 4 = severe narrowing of the joint space with many osteophytes. The articular appearance of macroscopic lesions was graded as follows: 0 = normal appearance; 1 = slight yellowish discoloration of the chondral surface; 2 = small cartilage erosions in load-bearing areas; 3 = large erosions extending down to the subchondral bone; and 4 = large erosions with large areas of subchondral bone exposed. chondral compartments (the femoral condyles, the tibial plateaus, the patella, and the femoral groove). All samples were measured by three assessors who were blinded to the induction procedure.

Histological analysis

Whole knee samples were fixed immediately in 4% paraformaldehyde, decalcified, embedded in paraffin, and cut into 4-μm tissue sections. Sections were stained with H&E and toluidine blue. The severity of OA lesions was graded according to modified Mankin scores[17]. The severity of OA lesions was scored based on proteoglycan (scale 0–4), collagen integrity (scale 0–3), cellular changes (scale 0–3), invasion of blood vessels (scale 0–1), bone modifications (scale 0–2) and cartilage structural changes (scale 0–5).

Immunohistochemistry

Resected knee samples were fixed immediately in 4% paraformaldehyde, decalcified, embedded in paraffin, and cut into 4-μm tissue sections. After antigen retrieval and blocking of endogenous peroxidase activity, sections were then incubated with primary antibodies (anti-LC3B, Abcam, Cambridge, MA, USA, 1:200 dilution) at 4°C overnight. A peroxidase-linked secondary antibody was then applied for 30 min at room temperature. Staining was detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Sections were then dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a coverslip using DePeX mounting medium.

Real time-PCR

Total RNA was removed from the articular cartilage using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into complementary DNA using a PrimeScript RT reagent kit (RR036A; Takara, Shiga, Japan). Expression of mRNA was evaluated by real-time PCR (RT-PCR) using SYBR® GreenER™ SuperMix (Takara) with specific primers for TRPV5 (Fw: 5'-CTGCCTGTGTGGGTAGTGAG-3' and Rv: 5'-GGTGAGTCCTTGGTTGTTGG-3'), LC3B (Fw: 5'-GTCGCTAACAAGCAGTGGGA-3' and Rv: 5'-AGGGCTTCTGGGGCTCTAAT-3'), calmodulin (Fw: 5'-GGCATCCTGCTTTAGCCTGAG-3' and RV: 5'-ACATGCTATCCCTCTCGTGTGA-3') and β-actin (Fw: 5'-CAGCCTTCCTTCCTGGGTATG-3' and Rv: 5'-TAGAGCCACCAATCCACACA-3'), and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The fold changes in gene expression were calculated using the following formula: $2^{-\Delta\Delta Ct}$ where ΔCt is the difference of the threshold cycle of the sample.

Isolation, culture and identification of rat primary chondrocytes

Normal articular cartilage was obtained from Sprague–Dawley rats. Articular cartilage pieces were incubated by sequential digestion with pronase (2 mg/mL) and collagenase D (1.5 mg/mL; Roche, Basel, Switzerland). Cells were cultured in 75 mm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. The medium used was DMEM (Gibco, Carlsbad, CA, USA) supplemented with L-glutamine (2 mM), gentamicin (0.5 g/mL), amphotericin B (0.5 g/mL), and 10% fetal calf serum (FCS; Gibco). Immunocytochemical analyses were performed to identify chondrocyte phenotype. Monolayer cells were incubated with anti-type II collagen antibody (Abcam, 1:400 dilution) at 4°C overnight. The secondary antibody was then applied for 30 min at room temperature. Staining was detected with DAB. Sections were then dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a coverslip using DePeX mounting medium.

Small interfering RNA transfection

Cells were transfected at 60–70% confluence using Lipofectamine™ 2000 (Invitrogen™) with TRPV5 siRNA (sc-42676; Santa Cruz Biotechnology, Dallas, TX, USA) at a final concentration of 100 nM according

to the manufacturers' instructions. Cells transfected with scrambled control siRNA (sc-37007; Santa Cruz Biotechnology) at 100 nM were used as a negative control. Cells were transfected for 72 h for the TRPV5 knockdown study, before Ca^{2+} measurements or western blot analysis.

Immunofluorescence staining

Per group chondrocytes in third generation were exposed to different concentrations of MIA (0, 1.5, 3.0, or 6.0 μM) or 6.0 μM MIA + 5 μM ruthenium red. However, TRPV5 siRNA transfected chondrocytes were exposed to 6.0 μM MIA for 12 h before experiments. Chondrocytes were incubated with primary antibody (Abcam; anti-LC3, 1:200 dilution and anti-calmodulin, 1:100 dilution) overnight at 4°C. The samples were then incubated with secondary antibody (Abcam, 1:100 dilution) for 1 h at 37°C. Chondrocyte nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Stained chondrocytes were observed under a fluorescence microscope, and images were captured as photographic records.

Determination of intracellular Ca^{2+}

The concentration of intracellular Ca^{2+} was determined using a green fluorescent dye, Fluo-4AM (Dojindo, Kumamoto, Japan). Chondrocytes were exposed to different concentrations of MIA (0, 1.5, 3.0, or 6.0 μM) or 6.0 μM MIA + 5 μM ruthenium red, and TRPV5 siRNA transfected chondrocytes were exposed to 6.0 μM MIA for 12 h before experiments. Cells were then incubated with 2 $\mu\text{mol/L}$ Fluo-4AM in HBSS for 30 min at 37 °C in the dark. Fluorescence images were obtained using an OLYMPUS IX71 inverted microscope and analyzed with MetaFluor software 7.8 (Molecular Devices, Sunnyvale, CA, USA). The ratio of fluorescence intensity ($\Delta F/F_0$) was used to compare intracellular Ca^{2+} concentrations under different treatments (F: average fluorescence intensity under different treatments, F_0 : control group fluorescence intensity without any intervention, $\Delta F = F - F_0$) [18].

Western blot analysis

Total proteins were extracted from treated cells, and concentrations were determined using a bicinchoninic acid reagent assay (Beyotime Biotechnology, Shanghai, China). Proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Blots were incubated with primary antibodies (Abcam) including anti-TRPV5 (1:2000 dilution), anti-calmodulin (dilution 1:1000), anti-p-CAMK II (dilution 1:2000), anti-p-Bcl-1 (dilution 1:2000), anti-LC3 (dilution 1:2000), anti-Bcl-2 (dilution 1:2000), anti- β -actin (dilution 1:10000) and anti-Bcl-1 (Cell signaling technology) (dilution 1:3000) and anti CAMK II (Cell signaling technology) (dilution 1:5000) overnight at 4°C; this was followed by incubation with secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at 37°C, and bands were visualized using an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Bartlesville, OK, USA). Bands were then quantified using a Gel Doc 2000 UV Gel imaging system (Bio-Rad, Hercules, CA, USA).

Co-immunoprecipitation

Chondrocytes were exposed to 6.0 μM MIA or 6.0 μM MIA + 5 μM ruthenium red, and TRPV5 siRNA transfected chondrocytes were exposed to 6.0 μM MIA for 12 h before experiments. Next, cells were washed once with PBS buffer and lysed in immunoprecipitation lysis buffer (Thomas Scientific Company). Antibodies to Beclin1 (Cell signaling technology) were added to the cell lysates and incubated for one hour at 4°C. Protein A agaroses (Santa Cruz Biochnology) were then added to the cell lysates 20 μl each, incubated for overnight at 4°C. After the immunoprecipitation, cell lysates were briefly spun and washed three times with PBS buffer. Immunoblotting was performed with Bcl-2 antibody to confirm the Beclin1 and Bcl-2 interactions.

Statistical analysis

All the experiments were performed in triplicate. All data results were expressed as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using SPSS 17.0 (IBM, Rochester, MN, USA). Differences between means were analyzed using one-way analysis of variance (ANOVA) with different times as the independent factors. A paired *t*-test was applied to evaluate differences between the maximum MIA dose group alone with the ruthenium red + MIA group and TRPV5 siRNA transfected chondrocytes, respectively. In all analyses, significance was set at $P = 0.05$.

Results

Effect of the TRPV5 inhibitor, ruthenium red, on OA progression in the MIA-induced rat OA model

Radiographic and macroscopic images showing evaluation scores of the knee joints from a rat OA model were assessed. In the radiographic images shown in Fig. 1B, obvious osteophytes and incomplete and thickened articular surfaces were observed in the OA group, especially with the maximum MIA dose. In the macroscopic evaluation of the OA group (Fig. 1C), the cartilage on the articular surface was thin and yellowish, with focal erosions of the tibial plateaus. Pathological features became increasingly worse as doses of MIA increased. However, the MIA + ruthenium red group showed markedly reduced pathological processes in both radiographic and macroscopic evaluations. Evaluation scores at varying time points of the MIA group were significantly different, according to the pathologic level of OA in radiographic and macroscopic images ($P < 0.05$ for all; Fig. 1D, 1E). As shown in Figure 2A, and 2B, Both hematoxylin and eosin (H&E) and toluidine blue staining revealed a major loss of chondrocytes, with the chondrocyte layer becoming thin and with few cells in the MIA group. Interestingly, the protective effect of ruthenium red against MIA-induced cartilage

Fig. 1. Macroscopic and radiographic analyses and the protective effect of the TRPV5 inhibitor (ruthenium red) in the MIA-induced rat OA model. (A) Flowchart representing groups of experimental animals (B) Representative X-ray photographs of total knee joints. (C) Macroscopic photographs of tibial plateaus of rat knee joints. (D) Radiographic scores measuring joint destruction. (E) Semi-quantitative macroscopic scores measuring joint destruction. Data are presented as mean $\pm$ SEM ($n = 3$). * $P < 0.05$ vs. untreated control; # $P < 0.05$ between MIA 21 days and MIA + ruthenium red treatment groups. # $P < 0.05$ vs. the MIA treatment group alone 21 days. TRPV5, transient receptor potential channel vanilloid 5; OA, osteoarthritis; MIA, monosodium iodoacetate; RR, ruthenium red.

Fig. 2. Evaluation of the protective effect of the TRPV5 inhibitor (ruthenium red) by histological analysis and down-regulated expression of autophagy marker LC3B correlated positively with the progression of osteoarthritis in an MIA-induced rat OA model. (A) Photomicrographs showing representative hematoxylin and eosin (H&E) staining of rat knee joints. (B) Photomicrographs showing representative toluidine blue staining of rat knee joints. (C) Photomicrographs showing representative immunohistochemical analyses of LC3B expression of the articular chondrocyte in each group. ($\times 40$ and $\times 400$ [inset] magnification for 2A, B and C). (D) Semi-quantitative Mankin scores for joint destruction. Data are presented as mean $\pm$ SEM ($n = 3$). * $P < 0.05$ vs. untreated control; # $P < 0.05$ between MIA 21 day and MIA + ruthenium red 21 day treatment groups. MIA, monosodium iodoacetate. RR, ruthenium red.

degradation was reversed by pretreatment with ruthenium red. Ruthenium red significantly suppressed Mankin scores, accordingly ($P < 0.05$; Fig. 2D). These results suggest that in articular chondrocytes, TRPV5 may participate in the development of OA. They also suggest that the inhibition of TRPV5 using ruthenium red is an effective therapeutic approach to reduce joint destruction in rat OA.

Down-regulated expression of autophagy marker LC3 correlated positively with the progression of osteoarthritis induced by MIA

LC3 protein in articular cartilage was immunolocalized by immunohistochemistry. Mild staining of LC3 was observed in moderately severe degenerative articular cartilage (MIA 7 days; Fig. 2C), while staining became progressively weaker with increased severity of OA (MIA 14 and 21 days; Fig. 2C and 2D). However, staining of LC3 protein, an autophagy marker, in the MIA + ruthenium red group was markedly higher than that in the MIA 21 days alone group (Fig. 2C), which suggests that ruthenium red promoted the expression LC3. The levels of LC3B mRNA (Fig. 3C) and extracted LC3 protein detected (Fig. 3D) were in accordance with IHC results for LC3, which were significantly down-regulated in OA cartilage. The ratio

Fig. 3. TRPV5 expression is up-regulated in the articular cartilage of MIA-induced OA and correlated positively with the expression of Ca^{2+} -binding protein, calmodulin, in OA articular cartilage. (A) Reverse transcription polymerase chain reaction (RT-PCR) showing the level of TRPV5 mRNA in OA from different MIA-stimulated groups compared to the normal group. $*P < 0.05$ vs. untreated control, $\#P > 0.05$ between MIA 21 day and MIA + ruthenium red 21 day treatment groups; (B) RT-PCR showing the level of calmodulin mRNA in OA from different stimulated groups compared to the normal group. $*P < 0.05$ vs. untreated control; $\#P < 0.05$ between MIA 21 day and MIA + ruthenium red 21 day treatment groups. (C) RT-PCR showing the level of LC3B mRNA in OA from different stimulated groups compared to the normal group. $*P < 0.05$ vs. untreated control, $\#P < 0.05$ between MIA 21 day and MIA + ruthenium red 21 day treatment groups. (D) TRPV5, calmodulin, LC3-I, and LC3-II protein expression from different stimulated groups at each time point as detected by Western blotting. (E) A bar graph showing the level of TRPV5, calmodulin, LC3-I, and LC3-II proteins in various treatment groups. $*P < 0.05$, $**P < 0.05$, $***P < 0.005$ vs. untreated control, $\#P < 0.05$, $##P < 0.05$, $###P < 0.05$, between MIA 21 day and the MIA + ruthenium red 21 day treatment groups. (F) The expression of LC3-II/LC3-I was shown by Western blotting. The optical density for LC3-II/LC3-I was analyzed for the various groups. $*P < 0.05$ vs. untreated control; $\#P < 0.05$ between MIA 21 day and MIA + ruthenium red 21 day treatment groups. Each column represents the mean $\pm$ SEM ($n = 3$). MIA, monosodium iodoacetate; RR, ruthenium red.

of protein LC3-II/LC3-I as the autophagy label decreased sharply but increased in the additional ruthenium red-treated group (Fig. 3E and F) and correlated positively with the degree of OA lesions ($P < 0.05$; Fig. 3F). In summary, these results suggest that intra-articular injection of ruthenium red can counter autophagy inhibition induced by MIA.

TRPV5 was expressed in normal articular cartilage, and its up-regulation correlated positively with calmodulin, a Ca^{2+} -binding protein, in OA articular cartilage

TRPV5 and calmodulin mRNA and protein were identified by real time polymerase chain reaction (RT-PCR) and western blot, respectively. In articular cartilage, TRPV5 was weakly expressed in normal cartilage while mild expression was observed in degenerative cartilage of moderate severity (MIA 7 and 14 days; Fig. 3A), accordingly. Up-regulated TRPV5

Fig. 4. Isolation, culture, identification and TRPV5 knockdown identification of rat primary chondrocytes. Primary chondrocyte culture after (A) 2 days, (B) 4 days (C) and 6 days. (D) Primary chondrocytes were stained with anti-collagen II as an indication of functional chondrocytes. (All photomicrographs are at $\times 40$ magnification and $\times 400$ magnification [inset]). (E) reduced mRNA Reverse transcription polymerase chain reaction (RT-PCR) showing the level of TRPV5 mRNA in TRPV5 siRNA groups compared to normal group, $*P < 0.005$. (F) TRPV5 protein expression from TRPV5 siRNA groups and normal group detected by Western blotting. (G) A bar graph showing the level of TRPV5 proteins in TRPV5 siRNA various normal (control) groups, $\#P < 0.005$.

expression was detected in the articular cartilage of rats with the most severe OA (MIA 21 days; Fig. 3A, 3D). The expression of the Ca^{2+} -dependent protein, calmodulin, surprisingly, correlated with that of TRPV5 positively (Fig. 3B, 3D). These results suggest that TRPV5 levels were significantly greater in OA cartilage, and the degree of up-regulation correlated positively with the degree of OA lesions ($P < 0.05$; Fig. 3F). However, the function of TRPV5 may correlate with the formation of the Ca^{2+} -calmodulin complex.

Morphological observation of primary rat chondrocytes, and LC3 and calmodulin expression in normal and MIA-stimulated chondrocytes

The morphology of rat primary chondrocytes, as observed under the microscope over time, initially resembled a pebble shape that became polygonal-shaped by day 6 (Fig. 4 A, B, C). Primary chondrocytes were stained with anti-collagen II as an indication of functional chondrocytes. We observed LC3B and calmodulin expression in chondrocytes *in vitro* by immunofluorescence staining. As shown in Fig. 5A, calmodulin protein was aggregated into large clumps, while LC3B (Fig. 5B) was aggregated into granules distributed in the perinuclear area of chondrocytes. A strong immunofluorescence staining of calmodulin in the MIA-stimulated group was noted, with the level of staining intensifying as MIA concentrations increased. However, calmodulin staining intensity was lower in both the ruthenium red group and in TRPV5 siRNA-transfected chondrocytes exposed to $6.0 \mu\text{M}$ MIA. However, surprisingly, the LC3B stain contrasted with that of calmodulin, which illustrates that autophagy-related proteins such as LC3B protein were inhibited with the increase in MIA concentration. Thus, TRPV5 expressed in chondrocytes may be linked to LC3B and calmodulin expression in experimental OA, which correlated with the degree of degeneration in chondrocytes.

Functional identification of up-regulated expression of TRPV5 mediates calcium influx overload in MIA-stimulated chondrocytes

Calcium influx in chondrocytes was studied by monitoring intracellular cytosolic Ca^{2+} fluorescence intensity using a Fluo-4AM stain. The fluorescence intensity in the MIA-

Fig. 5. Calmodulin and LC3B expression in untreated and MIA-stimulated chondrocytes. Expression of (A) calmodulin (red) and (B) LC3B (red) was determined by immunofluorescence staining (magnification $\times 200$). MIA, monosodium iodoacetate; RR, ruthenium red; DAPI, blue nuclear stain.

Fig. 6. Fluorescent images of rat primary chondrocytes labelled with the Ca^{2+} indicator dye Fluo-4AM (green). Chondrocytes were (A) not exposed to MIA (control) or exposed to (B) 1.5 μM (C) 3 μM (D) 6 μM MIA or (E) 6 μM MIA + 5 μM ruthenium red for 12 h. (F) TRPV5 siRNA transfected chondrocytes were exposed to 6 μM MIA for 12 h. (All photomicrographs are shown at $\times 200$ magnification). (G) Bar graph showing the level of relative fluorescent intensity of each group. $*P < 0.05$; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$. Each column represents mean $\pm$ SEM ($n = 3$). MIA, monosodium iodoacetate. RR, ruthenium red.

stimulated group gradually increased with increasing concentrations of MIA (0, 1.5, 3, 6 μM ; Fig. 6A, B, C, D) while the fluorescence intensity was significantly reduced by treating with 5 μM ruthenium red (Fig. 6E), or in TRPV5 siRNA-transfected chondrocytes (Fig. 6F) exposed to 6.0 μM MIA. These results indicate that TRPV5 has a specific role in mediating extracellular Ca^{2+} influx (Fig. 6G).

Fig. 7. Increase of calcium influx activates calmodulin that inhibits chondrocyte autophagy by activating the TRPV5- Ca^{2+} -CAMK II signaling pathway. (A) TRPV5, calmodulin, p-CAMK II, p-Beclin1, LC3-I, Bcl-2 and LC3-II protein expression from different MIA concentrations were detected by Western blotting. (B) A bar graph showing relative levels of TRPV5, calmodulin, p-CAMK II, p-Beclin1, LC3-II, and Bcl-2 proteins. $*P < 0.05$, $**P < 0.05$, $***P < 0.05$, $\#P < 0.05$, $\#\#P < 0.05$, $\#\#\#P < 0.05$ vs. untreated control. (C) LC3-II/LC3-I shown by Western blotting after optical density analysis. $*P < 0.05$ vs. untreated control. (D) TRPV5, calmodulin, p-CAMK II, p-Beclin1, LC3-I, LC3-II and Bcl-2 protein expression from different groups was detected by Western blotting. (E) LC3-II/LC3-I as shown by western blotting after optical density analysis. $*P < 0.05$, $\#P < 0.05$ vs. the untreated control group. Each column represents mean $\pm$ SEM ($n = 3$). (F) A bar graph showing the level of TRPV5, calmodulin, p-CAMK II, p-Beclin1, LC3-II and Bcl-2 proteins. $*P < 0.05$, $**P < 0.05$, $***P < 0.05$, $\#P < 0.05$, $\#\#P < 0.05$, $\#\#\#P < 0.05$, between 6 μM MIA treatment and untreated control groups; $*P < 0.05$, $**P < 0.05$, $***P < 0.05$, $\#P < 0.05$, $\#\#P < 0.05$, $\#\#\#P < 0.05$ between TRPV5 siRNA exposed to 6 μM MIA and untreated control groups. (G) Co-immunoprecipitation of Beclin 1 and Bcl-2 proteins from different groups. Representative blots show immunoprecipitation with anti-Beclin1 antibody. Immunocomplexes were subjected to immunoblotting and stained sequentially with anti-Bcl-2 antibody and anti-Beclin1 antibody as a control of precipitation. MIA, monosodium iodoacetate; RR, ruthenium red. p-CAMK II, phosphorylated calcium/calmodulin dependent protein kinase II; TRPV5, transient receptor potential channel vanilloid 5.

Increase of calcium influx activates calmodulin that inhibits chondrocyte autophagy by activating the TRPV5- Ca^{2+} -CAMKII signaling pathway

To explore the mechanism of how up-regulated TRPV5 inhibits chondrocyte autophagy, protein expression was determined by western blot. As shown in Fig. 7A, compared with the normal group, the expressions of calmodulin, p-CAMK II, p-Beclin1 and Bcl-2 became gradually up-regulated with an increase in MIA dose. However, LC3- II became gradually

down-regulated with an increase in MIA dose ($P < 0.05$; Fig. 7B). In addition, treating with ruthenium red and transfection with TRPV5 siRNA inhibited the TRPV5-mediated calcium influx overload observed in chondrocytes exposed to 6.0 μM MIA, compared with the 6 μM MIA only-induced group (Fig. 6E). Such pathway proteins all showed a corresponding decrease; however, LC3 become up-regulated slightly ($P < 0.05$; Fig. 7D and E). The ratio of protein LC3 II / LC3- I decreased sharply in 6 μM MIA (Fig. 7C), but increased in the additional ruthenium red treated or TRPV5 siRNA groups (Fig. 7F). In addition, to determine interactions between Beclin1 and Bcl-2 proteins, Co-immunoprecipitation using anti-Beclin1 antibody was performed. We found that Bcl-2 and Beclin1 could combined to form complex (Fig. 7G). In all Beclin 1-directed immunoprecipitates, the presence of Beclin 1 protein was confirmed. Likewise, the associations between Beclin 1 and Bcl-2 proteins were markedly reduced in the TRPV5 siRNA and Ruthenium red groups compared with MIA induced group. Thus up-regulated TRPV5 can stimulate calcium influx and overloading calcium can activate calmodulin and CAMK II proteins in the supposed pathway. CAMK II, in turn, can activate Beclin1 inhibition of chondrocyte autophagy and the combination of Bcl-2 and Beclin1 may further play a role in the autophagy inhibition.

Discussion

Osteoarthritis (OA) is a common joint disorder and a leading cause of disability worldwide. Based on current knowledge, autophagy, a self-protective mechanism in chondrocytes, has become a promising target to improve the viability and function of chondrocytes, thereby impeding the progress of OA [19, 20]. Autophagy may be activated in response to various stress stimuli, providing protective roles for chondrocytes [21]. Therefore, understanding the mechanism of inhibition of chondrocyte autophagy in OA is essential for developing appropriate targeted therapies for the treatment of this disease. An MIA-induced experimental OA rat model was used to imitate a model of articular cartilage degeneration in which to study the inhibition of chondrocyte autophagy. The advantages of the MIA-induced experimental OA model are that it is an easy and quick to set up model that shows dose- and time-dependent OA-like lesions, functional impairments similar to those observed in human disease [22].

TRPV5 is a member of the TRPV subfamily that is implicated in functions such as acting as a facilitative transporter of calcium. More recently, elevated TRPV5 expression has been found in calcium-containing tissue, including hepatocytes, osteoblasts, enterocytes/monocytes and colon epithelial cells. TRPV5 is also expressed in neurons [23] and non-neuronal cells such as smooth muscle cells [24]. Abnormal expression of TRPV5, can lead to diseases such as nephrosis syndrome in the kidney [25] and cause the abnormal resorption of bone in osteoblasts [26].

In our study, we have comprehensively shown that the expression of TRPV5 in cartilage and the up-regulation of TRPV5 participated in an OA process in the MIA-induced rat model. A direct linear correlation was observed between the expression (relative mRNA and protein expression levels) of TRPV5 with the OA process in our rat model. The levels of LC3B mRNA and protein detected by western blot were significantly down-regulated in OA cartilage, with the degree of down-regulation positively correlating with the degree of OA lesions, which contrasted with upregulated TRPV5 levels. LC3B protein in articular cartilage was weak staining in moderately severe degenerative cartilage, especially in the most severe OA articular cartilage. The intra-articular injection of ruthenium red countered the decreased LC3- II expression induced by MIA. The relationship between the up-regulated expression of TRPV5 and the inhibition of autophagy in cartilage chondrocytes is worthy of further exploration.

After discovering TRPV5 in chondrocytes, we then investigated mechanisms involved in the pathogenesis of OA that were related to Ca^{2+} influx overloading. We verified the functionality and activation of the TRPV5 channel in primary rat chondrocytes by measuring

Fig. 8. Diagram of the signaling cascade involved in the effect of TRPV5-mediated Ca^{2+} influx on chondrocyte autophagy inhibition. TRPV5 may exert a core initiation effect in mediating the inhibition of autophagy signaling via regulation of cytosolic Ca^{2+} accumulation.

Ca^{2+} influx using Fluo-4AM, and found that the fluorescence gradually increased with increasing MIA concentration. However, treatment with ruthenium red significantly decreased the fluorescence intensity of Fluo-4AM. Reduced Fluo-4AM intensity was also seen in TRPV5 siRNA transfected chondrocytes. This indicated that TRPV5 had a specific effect on the cytosolic Ca^{2+} concentration and on mediating extracellular Ca^{2+} influx.

The role of Ca^{2+} influx through Ca^{2+} -selective channels has increasingly been more extensively studied [11, 12]. Indeed, Ca^{2+} entry pathways have been identified in developing and mature chondrocytes, including N-methyl-D-aspartate receptor subunits [27], TRP channels and voltage-operated Ca^{2+} channels [28]. Of these pathways, TRPV is an important channel but has been less well studied. The TRPV4 ion channel is a key mediator of biomechanical signals and a significant component of chondrocyte mechanotransduction pathways [29]. The importance of Ca^{2+} in cell death has been emphasized, including in the production of calcium entry-dependent reactive oxygen species [30], and in mitochondrial depolarization and DNA fragmentation [31]. Recently, intracellular Ca^{2+} signaling was identified as having an important role in the regulation of autophagy [10].

We found that up-regulated TRPV5 can stimulate calcium influx and overloading calcium can result in the activation of the calcium sensor protein, calmodulin. The Ca^{2+} /calmodulin complex is a death-associated protein kinase which participates in CAMK II activation. Activated CAMK II then inhibits autophagy through phosphorylating Beclin1 and attenuating autophagosome formation. Interestingly, this inhibitory effect became more obvious with an increase in calcium ion influx. However, under conditions of pretreatment with ruthenium red or silencing TRPV5, the inhibition of autophagy by MIA became diminished or disappeared. Likewise, we also found that with the increase of intracellular calcium, the expression level of Bcl-2 also increased gradually accompanied with the up-regulation of TRPV5 in the osteoarthritis process. The increase of Bcl-2 level may be a "self-protection" of resistance to external injury in Ca^{2+} overloading condition [32, 33]. A recent study reported that Bcl-2, as an autophagy inhibitor could suppress chondrocyte autophagy and strengthen cartilage degradation in OA [34]. However, some studies have also shown that Beclin1, one of the autophagic proteins, interacts with anti-apoptotic Bcl-2 protein. Direct interaction between Beclin1 and Bcl-2 prevents cellular autophagy [35]. However, this effect has not been reported in the cartilage. In addition, we found that Bcl-2 and Beclin1 could combined to form complex accompanied by the decrease of autophagy marker LC3-II. Thus, we conclude that the combination of Bcl-2 and Beclin1 may further play a role in the autophagy inhibition, which may be a synergistic effect with phosphorylated Beclin1. The involvement of TRPV5 as an initiator of the inhibition of autophagy in chondrocytes in OA is outlined in Figure 8.

Conclusion

In summary, we found that the TRPV5 cation channel is functionally up-regulated in OA articular cartilage. Up-regulated TRPV5 may be an initiating factor that induces the inhibition of chondrocyte autophagy via the mediation of Ca^{2+} influx. These findings suggest an intriguing role for TRPV5 as a mediator and drug target in OA.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (General Program; No.: 81272050); and the Excellent PhD Program of Shengjing Hospital of China Medical University (No.: ME33).

Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Thomas E, Peat G, Croft P: Defining and mapping the person with osteoarthritis for population studies and public health. *Rheumatology (Oxford)* 2014;53:338-345.
- 2 Wei Y, Bai L: Recent advances in the understanding of molecular mechanisms of cartilage degeneration, synovitis and subchondral bone changes in osteoarthritis. *Connect Tissue Res* 2016;10.1080/03008207.2016.11770361-17.
- 3 Sasaki H, Takayama K, Matsushita T, Ishida K, Kubo S, Matsumoto T, Fujita N, Oka S, Kurosaka M, Kuroda R: Autophagy modulates osteoarthritis-related gene expression in human chondrocytes. *Arthritis Rheum* 2012;64:1920-1928.
- 4 Chen Z, Jin T, Lu Y: AntimiR-30b Inhibits TNF-alpha Mediated Apoptosis and Attenuated Cartilage Degradation through Enhancing Autophagy. *Cell Physiol Biochem* 2016;40:883-894.
- 5 Shin YJ, Han SH, Kim DS, Lee GH, Yoo WH, Kang YM, Choi JY, Lee YC, Park SJ, Jeong SK, Kim HT, Chae SW, Jeong HJ, Kim HR, Chae HJ: Autophagy induction and CHOP under-expression promotes survival of fibroblasts from rheumatoid arthritis patients under endoplasmic reticulum stress. *Arthritis Res Ther* 2010;12:R19.
- 6 Lotz MK, Carames B: Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. *Nat Rev Rheumatol* 2011;7:579-587.
- 7 Cheng NT, Guo A, Meng H: The protective role of autophagy in experimental osteoarthritis, and the therapeutic effects of Torin 1 on osteoarthritis by activating autophagy. *BMC Musculoskelet Disord* 2016;17:150.
- 8 Carames B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M: Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. *Arthritis Rheum* 2010;62:791-801.
- 9 Park JH, Choi SH, Kim H, Ji ST, Jang WB, Kim JH, Baek SH, Kwon SM: Doxorubicin Regulates Autophagy Signals via Accumulation of Cytosolic Ca^{2+} in Human Cardiac Progenitor Cells. *Int J Mol Sci* 2016;17:
- 10 Decuypere JP, Kindt D, Luyten T, Welkenhuyzen K, Missiaen L, De Smedt H, Bultynck G, Parys JB: mTOR-Controlled Autophagy Requires Intracellular Ca^{2+} Signaling. *PLoS One* 2013;8:e61020.
- 11 Sun Z, Han J, Zhao W, Zhang Y, Wang S, Ye L, Liu T, Zheng L: TRPV1 activation exacerbates hypoxia/reoxygenation-induced apoptosis in H9C2 cells via calcium overload and mitochondrial dysfunction. *Int J Mol Sci* 2014;15:18362-18380.
- 12 Lee KP, Nair AV, Grimm C, van Zeeland F, Heller S, Bindels RJ, Hoenderop JG: A helix-breaking mutation in the epithelial Ca^{2+} channel TRPV5 leads to reduced Ca^{2+} -dependent inactivation. *Cell Calcium* 2010;48:275-287.
- 13 Matsumoto N, Ezaki J, Komatsu M, Takahashi K, Mineki R, Taka H, Kikkawa M, Fujimura T, Takeda-Ezaki M, Ueno T, Tanaka K, Kominami E: Comprehensive proteomics analysis of autophagy-deficient mouse liver. *Biochem Biophys Res Commun* 2008;368:643-649.

- 14 Marshall CB, Nishikawa T, Osawa M, Stathopulos PB, Ikura M: Calmodulin and STIM proteins: Two major calcium sensors in the cytoplasm and endoplasmic reticulum. *Biochem Biophys Res Commun* 2015;460:5-21.
- 15 Gu J, Law AY, Yeung BH, Wong CK: Activation of gill Ca^{2+} -sensing receptor as a protective pathway to reduce Ca^{2+} -induced cytotoxicity. *J Mol Endocrinol* 2014;53:155-164.
- 16 Montell C: Physiology, phylogeny, and functions of the TRP superfamily of cation channels. *Sci STKE* 2001;2001:re1.
- 17 Liu Y, Wu D, Song F, Zhu C, Hui Y, Zhu Q, Wu J, Fan W, Hu J: Activation of $\alpha 7$ nicotinic acetylcholine receptors prevents monosodium iodoacetate-induced osteoarthritis in rats. *Cell Physiol Biochem* 2015;35:627-638.
- 18 Sun R, Yang Y, Ran X, Yang T: Calcium Influx of Mast Cells Is Inhibited by Aptamers Targeting the First Extracellular Domain of Orai1. *PLoS One* 2016;11:e0158223.
- 19 Takayama K, Kawakami Y, Kobayashi M, Greco N, Cummins JH, Matsushita T, Kuroda R, Kurosaka M, Fu FH, Huard J: Local intra-articular injection of rapamycin delays articular cartilage degeneration in a murine model of osteoarthritis. *Arthritis Res Ther* 2014;16:482.
- 20 Carames B, Hasegawa A, Taniguchi N, Miyaki S, Blanco FJ, Lotz M: Autophagy activation by rapamycin reduces severity of experimental osteoarthritis. *Ann Rheum Dis* 2012;71:575-581.
- 21 Shapiro IM, Layfield R, Lotz M, Settembre C, Whitehouse C: Boning up on autophagy: the role of autophagy in skeletal biology. *Autophagy* 2014;10:7-19.
- 22 Guingamp C, Gegout-Pottie P, Philippe L, Terlain B, Netter P, Gillet P: Mono-iodoacetate-induced experimental osteoarthritis: a dose-response study of loss of mobility, morphology, and biochemistry. *Arthritis Rheum* 1997;40:1670-1679.
- 23 Lang F, Strutz-Seebohm N, Seebohm G, Lang UE: Significance of SGK1 in the regulation of neuronal function. *J Physiol* 2010;588:3349-3354.
- 24 Earley S: Vanilloid and melastatin transient receptor potential channels in vascular smooth muscle. *Microcirculation* 2010;17:237-249.
- 25 Nie M, Bal MS, Yang Z, Liu J, Rivera C, Wenzel A, Beck BB, Sakhaee K, Marciano DK, Wolf MT: Mucin-1 Increases Renal TRPV5 Activity In Vitro, and Urinary Level Associates with Calcium Nephrolithiasis in Patients. *J Am Soc Nephrol* 2016;10.1681/asn.2015101100
- 26 Chamoux E, Bisson M, Payet MD, Roux S: TRPV-5 mediates a receptor activator of NF-kappaB (RANK) ligand-induced increase in cytosolic Ca^{2+} in human osteoclasts and down-regulates bone resorption. *J Biol Chem* 2010;285:25354-25362.
- 27 Matta C, Zakany R: Calcium signalling in chondrogenesis: implications for cartilage repair. *Front Biosci (Schol Ed)* 2013;5:305-324.
- 28 Fodor J, Matta C, Olah T, Juhasz T, Takacs R, Toth A, Dienes B, Csernoch L, Zakany R: Store-operated calcium entry and calcium influx via voltage-operated calcium channels regulate intracellular calcium oscillations in chondrogenic cells. *Cell Calcium* 2013;54:1-16.
- 29 Phan MN, Leddy HA, Votta BJ, Kumar S, Levy DS, Lipshutz DB, Lee SH, Liedtke W, Guilak F: Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes. *Arthritis Rheum* 2009;60:3028-3037.
- 30 Kovac S, Domijan AM, Walker MC, Abramov AY: Seizure activity results in calcium- and mitochondria-independent ROS production via NADPH and xanthine oxidase activation. *Cell Death Dis* 2014;5:e1442.
- 31 Llorente-Folch I, Rueda CB, Pardo B, Szabadkai G, Duchen MR, Satrustegui J: The regulation of neuronal mitochondrial metabolism by calcium. *J Physiol* 2015;593:3447-3462.
- 32 Pinton P, Ferrari D, Magalhaes P, Schulze-Osthoff K, Di Virgilio F, Pozzan T, Rizzuto R: Reduced loading of intracellular Ca^{2+} stores and downregulation of capacitative Ca^{2+} influx in Bcl-2-overexpressing cells. *J Cell Biol* 2000;148:857-862.
- 33 Giorgi C, Romagnoli A, Pinton P, Rizzuto R: Ca^{2+} signaling, mitochondria and cell death. *Curr Mol Med* 2008;8:119-130.
- 34 Zhang F, Wang J, Chu J, Yang C, Xiao H, Zhao C, Sun Z, Gao X, Chen G, Han Z, Zou W, Liu T: MicroRNA-146a Induced by Hypoxia Promotes Chondrocyte Autophagy through Bcl-2. *Cell Physiol Biochem* 2015;37:1442-1453.
- 35 Denton D, Xu T, Kumar S: Autophagy as a pro-death pathway. *Immunol Cell Biol* 2015;93:35-42.