Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2309-2323
DOI: 10.1159/000495874
Published online: 7 December 2018
Accepted: 29 November 2018

Original Paper

The Transient Receptor Potential Channel, Vanilloid 5, Induces Chondrocyte Apoptosis via Ca^{2+} CaMKII–Dependent MAPK and Akt/mTOR Pathways in a Rat Osteoarthritis Model

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Key Words
TRPV5 • Ca^{2+} • P-CaMKII • Chondrocyte apoptosis • Osteoarthritis

Abstract
Background/Aims: Chondrocyte apoptosis is a central pathological feature of cartilage in osteoarthritis (OA). Accumulating evidence suggests that calcium ions (Ca^{2+}) are an important regulator of apoptosis. Previously, we reported that the transient receptor potential channel vanilloid (TRPV5) is upregulated in monoiodoacetic acid (MIA)-induced OA articular cartilage. ***Methods:*** The protein levels of TRPV5, phosphorylated Ca^{2+}/calmodulin-dependent kinase II (p-CaMKII), and total CaMKII were detected *in vivo* using western blotting techniques. Primary chondrocytes were isolated and cultured *in vitro*. Then, p-CAMKII was immunolocalized by immunofluorescence in chondrocytes. Fluo-4AM staining was used to assess intracellular Ca^{2+}. Annexin V-fluorescein isothiocyanate / propidium iodide flow cytometric analysis was performed to determine chondrocyte apoptosis. Western blotting techniques were used to measure the expression of apoptosis-related proteins. ***Results:*** We found that ruthenium red (aTRPV5inhibitor)or(1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperaze (KN-62) (an inhibitor of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) phosphorylation) can relieve or even reverse OA *in vivo*. We found that TRPV5 has a specific role in mediating extracellular Ca^{2+} influx leading to chondrocyte apoptosis *in vitro*. The apoptotic effect in chondrocytes was inhibited by KN-62. We found that activated p-CaMKII could elicit the phosphorylation of extracellular signal-regulated protein kinase 1/2, c-Jun N-terminal kinase, and p38, three important regulators of the mitogen-activated protein kinase (MAPK) cascade. Moreover, we also showed that activated p-CaMKII could elicit the phosphorylation of protein kinase B (Akt) and two important downstream regulators of

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mammalian target of rapamycin (mTOR): 4E-binding protein, and S61 kinase. ***Conclusion:*** Our results demonstrate that upregulated TRPV5 may be an important initiating factor that activates CaMKII phosphorylation via the mediation of Ca^{2+} influx. In turn, activated p-CaMKII plays a critical role in chondrocyte apoptosis via MAPK and Akt/mTOR pathways.

Introduction

Osteoarthritis (OA) is a common, debilitating disease with a large societal and economic burden that leads to physical and psychological sequelae in the elderly population. Knee involvement, in the form of pain and stiffness, occurs more frequently than for other joints in OA, causing the greatest burden to the population, and often leads to significant disability requiring surgical intervention [1]. Pathologically, OA is characterized by the progressive degeneration of articular cartilage [2]. The cartilage becomes hypocellular in OA and is often accompanied by lacunar emptying, suggesting that chondrocyte apoptosis is a central feature in OA progression [3]. Thus, finding a cause for chondrocyte apoptosis during OA may become a potential and urgent strategy against this disease.

It is widely accepted that calcium ions (Ca^{2+}) are major intracellular second messengers and play an important role in apoptosis. The disruption of Ca^{2+} homeostasis, due to its sustained elevation in the cytoplasm, can trigger apoptosis [4]. The increase in intracellular calcium levels can result in the activation of the calcium sensor protein, calmodulin (CaM), and the combination of target proteins to form a Ca^{2+}/calmodulin complex [5]. Our study highlights how an increase in Ca^{2+} influx through Ca^{2+}-selective channels, such as the transient receptor potential channel vanilloid 5 (TRPV5) [6], can accelerate OA progression by activating calcium/calmodulin-dependent protein kinase II (CAMKII) [7]. Our findings also indicate that TRPV5 has a specific effect on the cytosolic Ca^{2+} concentration and cascade events of OA pathophysiology.

CaMKII, a Ser/Thr specific protein kinase, is a general integrator of Ca^{2+} signaling. CaMKII is activated in the presence of Ca^{2+} and CaM, which leads to autophosphorylation, generating a Ca^{2+}/CaM-independent form of the enzyme [8]. Increasing evidence indicates an elevation in cytoplasmic Ca^{2+} levels activates the mitogen-activated protein kinase (MAPK) cascade. Three distinct groups of MAPKs, including extracellular signal-regulated protein kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK, are also involved in chondrocyte apoptosis and cartilage degeneration in a rat osteoarthritis model [9-11].

Numerous studies have demonstrated that the development of Ca2+ can also be critical for the amino acid–mediated activation of mammalian target of rapamycin (mTOR) [12]. A serine/threonine (Ser/Thr) protein kinase, mTOR regulates differentiation, development, and survival in various cell types. Specifically, protein synthesis is regulated by mTOR via the phosphorylation and inactivation of translational repressor 4E-binding protein (4E-BP1), and through the phosphorylation and activation of S6 kinase (S6K1) [13]. The overexpression of mTOR is observed in human OA cartilage as well as in mouse experimental OA. Upregulated mTOR expression corresponds to increased chondrocyte apoptosis during OA [14]. However, evidence is lacking for a specific mechanism by which mTOR and MAPK signaling influence OA pathophysiology. This prompted us to study whether p-CaMKII activates the MAPK and mTOR pathways to trigger chondrocyte apoptosis via elevated [Ca^{2+}] influx through TRPV5 in OA.

Materials and Methods

Animals and Development of MIA-Induced Rat OA Models

Male Sprague-Dawley Rats (2 months old, 220-230 g in weight) were used. All rats were housed in groups of five per cage under standard laboratory conditions with free access to food and water, and a constant room temperature (22°C) and humidity (45% to 50%). Rats were randomly divided into groups

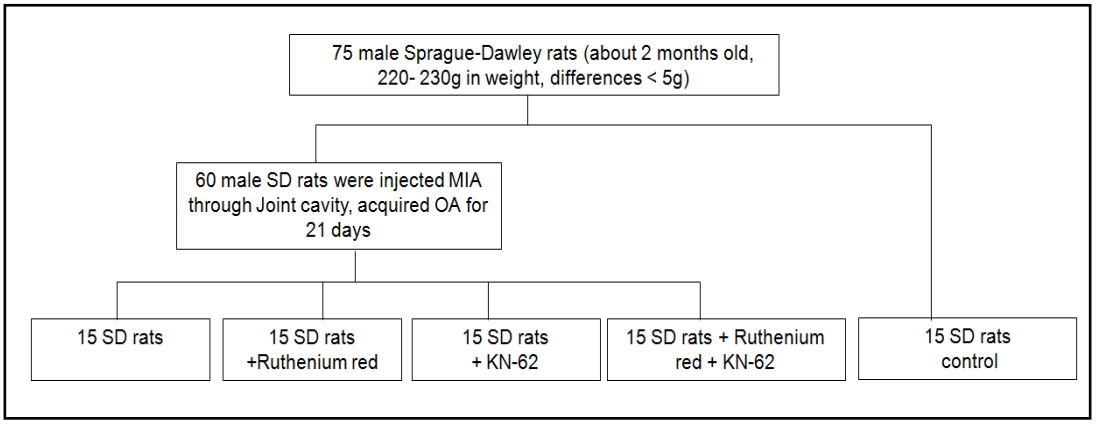

Fig. 1. Experimental animals grouped flowchart.

as described below (Fig. 1). Rats were given an intra-articular injection of MIA and ruthenium red (RR, Sigma USA) through the infra-patella ligament of left knee, at a dose of 1 mg in 50 µl sterile saline. KN62 were dissolved in Dimethyl sulfoxide (DMSO) then diluted with sterile saline at a concentration of 5 mg/ml were administrated intra-articularly 50µl. Control (normal) animals were given an injection of equi-volume sterile saline.

Macroscopic Analysis

Joint space was monitored using the digital X-ray (MX-20, Faxitron X-Ray Corp., Wheeling, IL, US). 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; 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; 4 = large erosions with large areas of subchondral bone exposure. Each of the chondral compartments (the femoral condyles, the tibial plateaus, the patella, and the femoral groove). All scores were given by three trained assessors who were blinded to the X-rays and macroscopic lesions analysis procedure.

Histological analysis and Immunohistochemistry (IHC)

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. Immunohistochemistry staining was performed by procedure. antigen retrieval and blocking of the endogenous peroxidase activity, sections were then incubated with anti-CaMKII (phospho T286) antibody (ab32678) (Abcam, USA, 1:150 dilution) at 4°C overnight. Then the secondary antibody(zhongshanjinqiao, China) was applied for 30 min at room temperature. Staining was detected with DAB (3, 3' -diaminobenzidine tetrahydrochloride).

Isolation, culture and identification of rat primary chondrocytes

Primary chondrocytes were isolated from rats as described [7]. Fresh medium was replaced every 2 days and chondrocytes reached approximately 80% confluence by days 4-5 as the P0 generation. Converged chondrocytes were then detatched with trypsin for subculture continually as generations P1, P2, and P3. Cells were used for experiments within the P3 generation. Immunocytochemistry was performed to identify chondrocyte phenotypes. Monolayer cells were incubated with anti-type II collagen antibody (Abcam, Cambridge, USA;1:400 dilution).

Immunofluorescence Staining

Chondrocytes were seeded at a density of 1×106 cells/well in completed growth medium in a 6-well plate for 24h respectively. Next day, three groups were pre-incubation with ruthenium red (10 µM), KN-62 (10 µM) and ruthenium red (10 µM) + KN-62 (10 µM) respectively for 30 minutes before 6 µM MIA incubation. The each well is at a final volume of 2ml completed growth medium. Cells were treated with MIA

for 12 h. Treated cells were then fixed in neutral formalin-buffered solution for 30 minutes, washed 3 times with PBS following and incubated with primary anti-CaMKII (phospho T286) antibody (ab32678) (Abcam, USA, 1:100 dilution) overnight at 4°C. Samples were then incubated with secondary antibody (Abcam, USA, 1:100 dilution) for 1 h at 37°C. The chondrocyte nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) for 5 min. The stained chondrocytes were observed under a fluorescence microscope.

Determination of intracellular Ca^{2+}

The concentration of intracellular Ca^{2+} was determined using a green fluorescent dye, Fluo-4AM (Dojindo, Kumamoto, Japan). The chondrocytes were grouped like in Immunofluorescence Staining method above. Cells were treated with MIA for 12 h and washed 3 times with D-Hanks balanced salt solution without Ca^{2+}. Subsequently, cell were loaded with 2 μmol/l Fluo-4AM (Dojindo, Japan) for 30 min at 37°C in the dark, then washed twice with D-Hanks balanced salt solution without Ca^{2+} to remove the extracellular Fluo-4/AM. Imaging was performed using an OLYMPUS IX71 inverted microscope and analyzed with Image-Pro Plus 6.0. The measured average fluorescence intensity of each cell in the field (F) normalized with the non-specific background fluorescence (F_0) to obtain the fluorescence intensity (F/F0) [15]. Statistical data are provided as percentage variation of treatments group release vs control (0 μM MIA).

Detection of Apoptosis by Flow Cytometry

MIA-induced apoptosis of chondrocytes was detected using an annexin V-FITC apoptosis detection kit (KeyGEN, China). The chondrocytes were grouped like in Immunofluorescence Staining method above. Cells were treated with MIA for 12 h and resuspended in 500 μl of binding buffer (KeyGEN, China), followed by incubation with 5 μl annexin V-FITC and 5 μl of propidium iodide (PI) at room temperature for 15 min in the dark. Flow cytometry with cell Quest software (BD Biosciences, San Jose, CA) was carried out.

Western blotting

Western blotting was performed as described [7] Total proteins were extracted from treated and tissues, 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 including Anti-TRPV5 antibody (ab77351) (Abcam, USA, 1:2000 dilution), anti-CaMKII antibody (ab52476) (Abcam, USA, 1:1000 dilution), anti-phospho -CaMKII antibody (ab32678) (Abcam, USA, 1:1000 dilution), anti–β-actin (ab8226) (Abcam, USA, 1:10000 dilution). Following antibodies were purchased from Cell Signaling Technology (Inc., Beverly, MA, USA) anti-phospho-JNK (#9255) (dilution 1:2000), anti-JNK (#9252) (dilution 1:1500), anti-phospho-p38 (#9215) (dilution 1:1000), anti-p38 (#9212) (dilution 1:1000), anti--phospho-Erk (#9106) (dilution 1:2000), anti-Erk(#9102) (dilution 1:2000), anti-phospho-Akt (#4051) (dilution 1:1000), and anti-Akt (#9272) (dilution 1:2000) anti-phospho-S6k1 (#9205) (dilution 1:2000), anti-S6k1 (#9202) (dilution 1:1500), anti-4E-BP1 (#9452) (dilution 1:2000), anti-Phospho-4E-BP1(#2855) (dilution 1:1000).

Statistical Analysis

All experiments in this study were repeated three times. Quantitative analysis of the bands was performed with the Image J analysis software (Version 1.30v; Wayne Rasband, NIH, USA). All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the results was carried out by paired t-test analysis. All statistical analyses were performed using SPSS 17.0 (IBM, Armonk, NY, USA). Significance was set at P = 0.05 for all statistical analyses.

Results

Effects of ruthenium red or KN62 treatment can delay OA progression in the MIA-induced rat OA model

To explore whether ruthenium red (RR) and KN62 have protective roles for cartilage in MIA-induced OA, OA changes of the knee at 21 days reperfusion with MIA were examined by radiography and macroscopic examination. Radiography (Fig. 2A) revealed that the normal

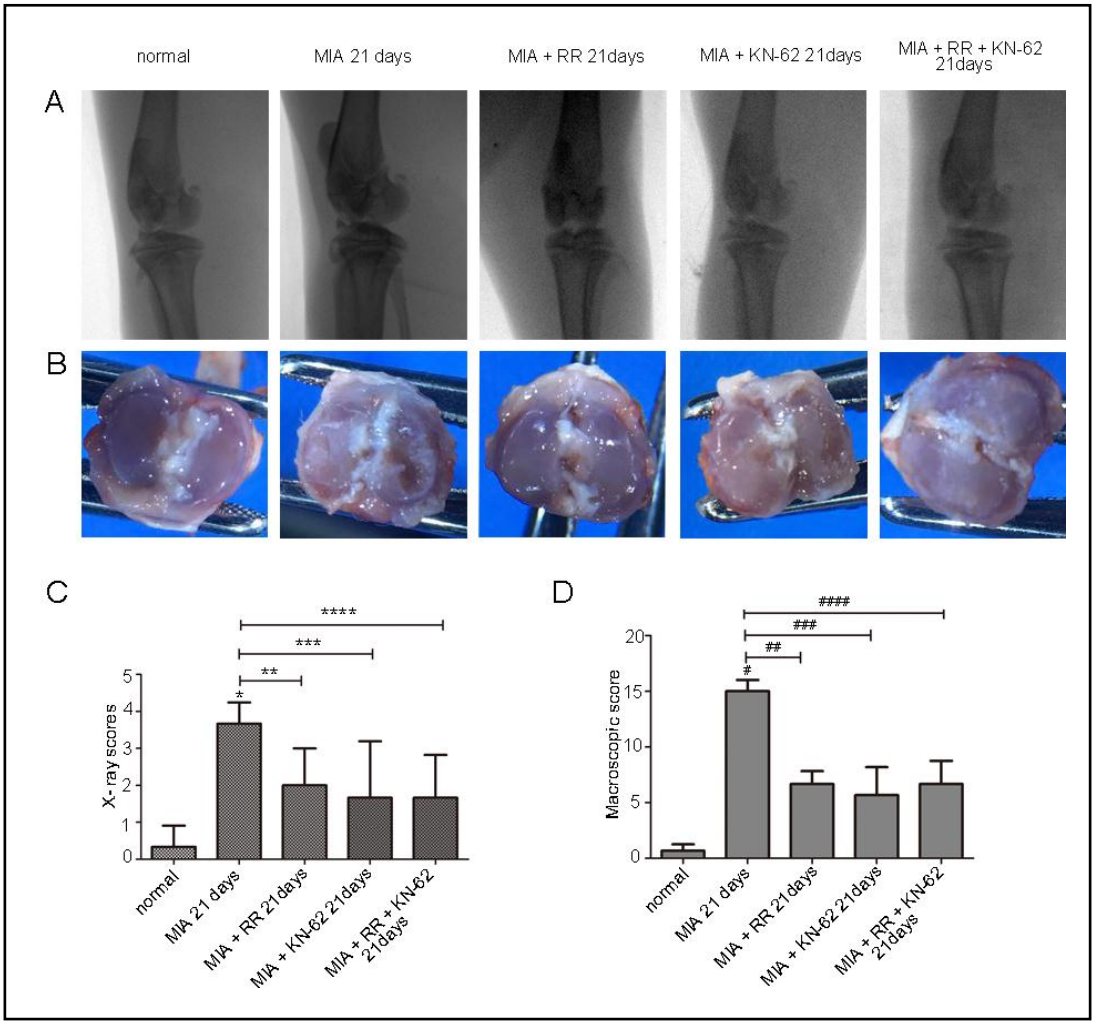

Fig. 2. Macroscopic and radiographic analyses and effects of ruthenium red or KN62 treatment can delay OA progression in the MIA-induced rat OA model. (A) Macroscopic photographs of tibial plateaus of the rat knee joints (B) X-ray photographs of the total knee joints (C) Radiographic scores measuring joint destruction (D) Macroscopic scores measuring joint destruction. Data are presented as mean ± SEM (n = 3). *P<0.05 vs. control; **P<0.05, ***P<0.05, ****P<0.05 vs. 6 μM MIA without therapy treatment. #P<0.05vs. control; ##P< 0.05, ###P< 0.05, ####P< 0.05 vs. 6 μM MIA without therapy treatment. MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine.

(control) group of rats had knee joints with a smooth surface. In contrast, obvious osteophytes as well as incomplete and thickened articular surfaces were observed in the knees of rats of the MIA 21 days group. However, treatment with ruthenium red, KN62, or ruthenium red combined with KN62 led to markedly less osteophytes and reduced pathological processes as observed in radiographic images of in rat knees. Macroscopic assessment revealed (Fig. 2B) MIA treatment resulted in marked cartilage erosion with large gray and losing its gloss areas even cartilage exfoliation. Changes in the subchondral bone was also explored 21 days after MIA injection; treatment with ruthenium red or KN-62 dramatically decreased cartilage degeneration induced by MIA injection. At the same time, treatment with ruthenium red combined with KN-62 showed the same protective effect as ruthenium red or KN-62 alone. Evaluation scores were consistent with the pathologic level of OA in radiographic images and macroscopic examination (Fig. 2C, 2D). These results suggest that in articular chondrocytes, the inhibition of TRPV5 using ruthenium red or the inhibition of CaMKII phosphorylation by KN-62 may be protective against the development of OA; the protective mechanisms

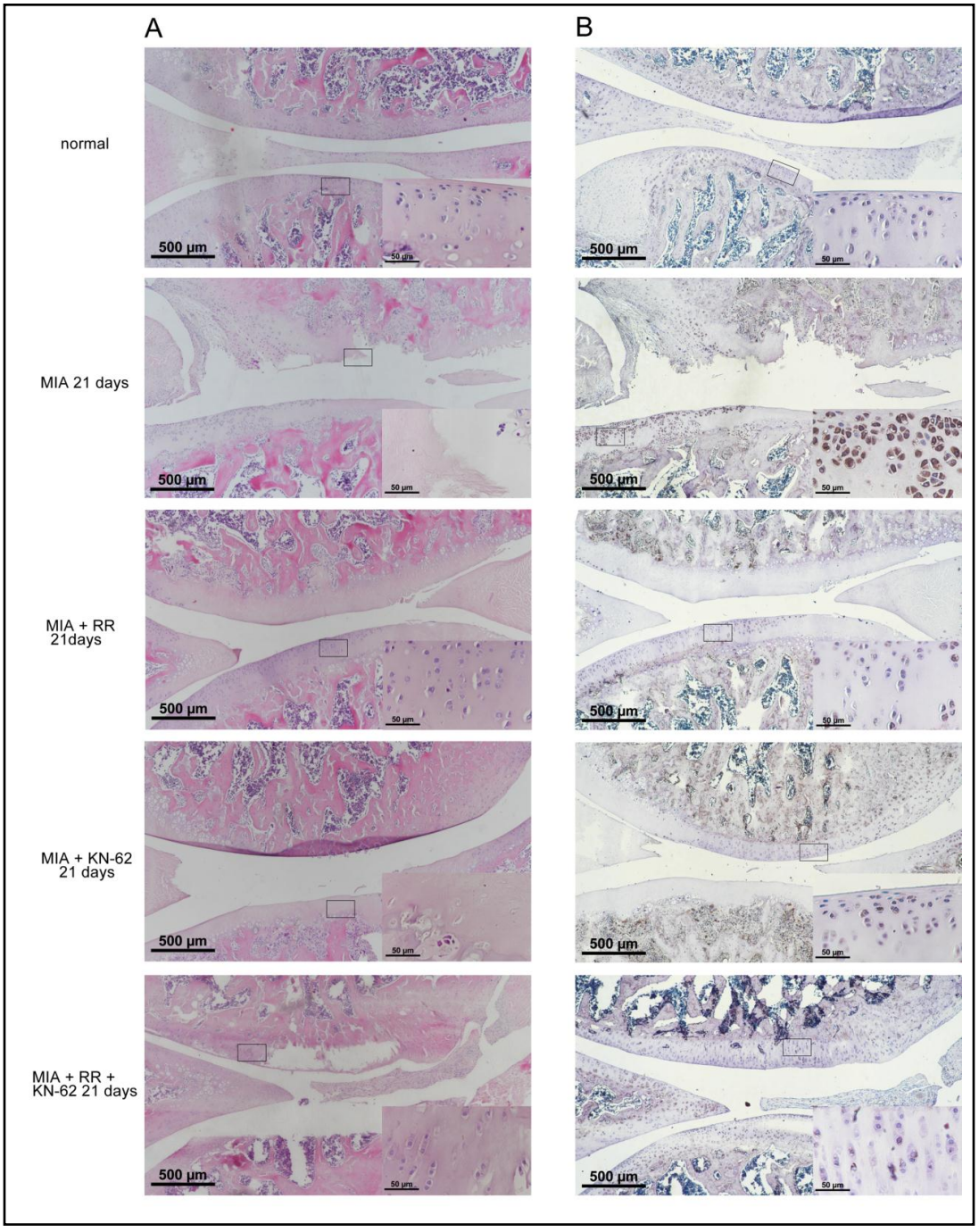

Fig. 3. Evaluation of the protective effect of the TRPV5 inhibitor (ruthenium red) and CAMKII phosphorylation inhibitor (KN-62) by histological analysis and expression of p-CAMKII correlated positively with the progression of osteoarthritis in an MIA-induced rat OA model. (A) Photomicrographs showing representative hematoxylin and eosin (H&E) staining of rat knee joints. (B) Photomicrographs showing representative immunohistochemical analyses of p-CAMKII expression of the articular chondrocyte in each group. Brown staining indicates specific p-CAMKII protein, and blue staining indicates the nucleus. The distribution of brown staining is positively correlated with p-CAMKII protein expression. (×40 and ×400 [inset] magnification for 2A, B and C). TRPV5, transient receptor potential channel vanilloid 5; MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; p-CAMKII, Ca2+/Calmodulin-Dependent Kinase II phosphorylation.

involved may be related to TRPV5 and CaMKII phosphorylation.

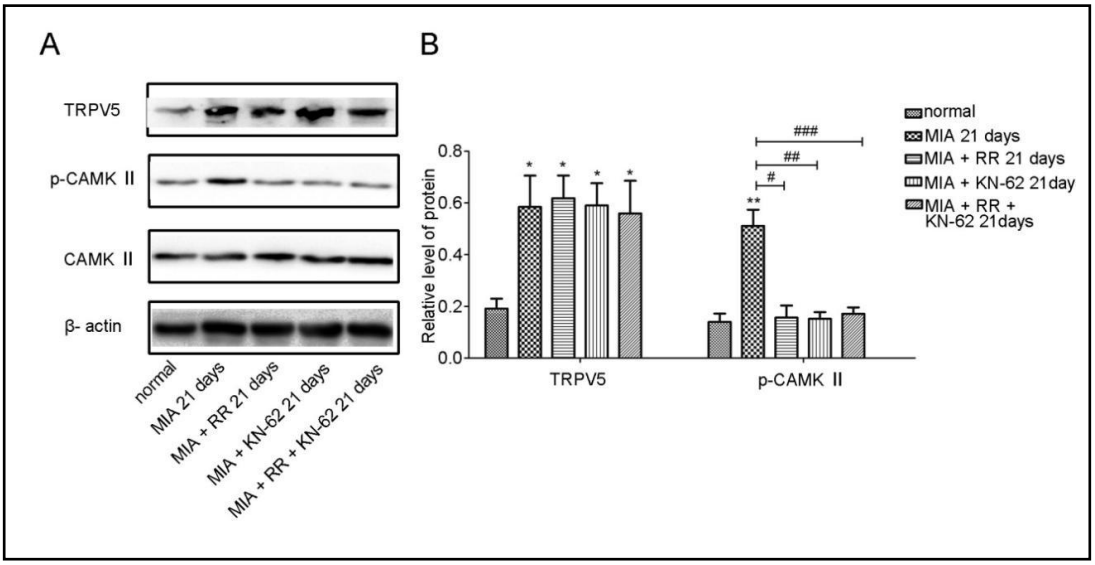

Fig. 4. TRPV5 and p-CAMKII protein expression in the articular cartilage of different group (A) TRPV5, p-CAMKIII protein expression from different stimulated groups at each time point as detected by Western blotting. (B) A bar graph showing the level of TRPV5, p-CAMKII proteins in various groups. *P<0.05 vs. untreated control, **P<0.05, #P< 0.05, ##P< 0.05, ###P< 0.05, #P<0.05, ##P<0.05, ###P<0.05 vs. MIA 21 day. Each column represents the mean ± SEM (n = 3). MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; p-CAMKII, Ca2+/ Calmodulin-Dependent Kinase II phosphorylation.

Activation of CAMKII phosphorylation correlated positively with TRPV5 function in parallel with the degree of osteoarthritis lesions

HE staining detected the severity of OA in different groups on histological analysis. In a comparison with the control group, the cartilage layer showed thinning, with a major loss of chondrocytes and lacunar emptying in the MIA 21 days group; cartilage block exfoliation even appeared (Fig. 3A). However, such cartilage degeneration was relieved and even reversed by treatment with RR and KN-62. These results suggest that treatment with both RR and KN-62 can change the process of osteoarthritis.

Interestingly, p-CAMKII protein, immunolocalized by immunohistochemistry in articular cartilage, was positively associated with the corresponding OA lesion pathology. Light staining of p-CAMKII was observed in normal articular cartilage (Fig. 3B), while staining intensity increased greatly after MIA 21days. However, at the same time, staining of p-CAMKII was markedly weaker after MIA 21days, after treatment with RR and KN-62. TRPV5, p-CAMKII, and CAMKII proteins were quantified by western blotting; the expression of TRPV5 and p-CAMKII increased by MIA 21 day (Fig. 4). RR and KN-62 treatment reduced the phosphorylation level of CAMKII protein; however, TRPV5 and total CAMKⅡ protein remained unchanged. These results indicate that upregulated p-CAMKII in the OA lesion and upregulated CAMKII phosphorylation was inhibited by KN62 and ruthenium red. These results also suggest channel function mediated by TRPV5 is related to the activation of CAMKⅡ phosphorylation, leading to the initiation of osteoarthritis.

Increased calcium in chondrocytes mediated by TRPV5 activated CAMKII phosphorylation

Primary chondrocytes isolated from rats were used *in vitro*. The calcium increase mediated by TRPV5 in chondrocytes was studied by monitoring intracellular cytosolic Ca^{2+} fluorescence intensity using a Fluo-4AM stain. The fluorescence intensity for the 6 μM MIA alone group was markedly higher than that of the 0 μM MIA group, while the fluorescence intensity was significantly reduced by treating with 10 μM ruthenium red or 10 μM ruthenium red + 10 μM KN-62 (Fig. 5A). However, for the KN-62 treatment alone group,

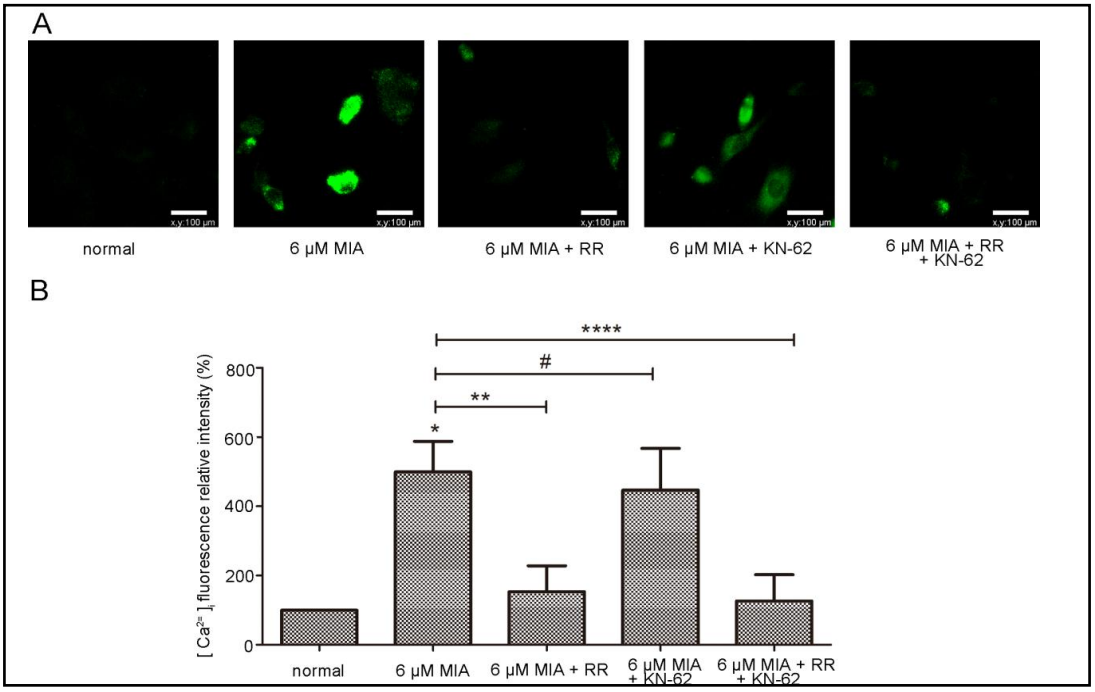

Fig. 5. Fluorescent images of rat primary chondrocytes labelled with the Ca2+ indicator dye Fluo-4AM (A) Ca^{2+} fluorescence relative intensity in different chondrocyte treatment groups (all photomicrographs are shown at ×200 magnification). (B) Bar graph showing the level of relative fluorescent intensity in each group. *P<0.05; **P<0.05; ****P<0.05; #P >0.05; Each column represents mean ± SEM (n = 3). MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; p-CAMK, Ca2+/Calmodulin-Dependent Kinase II phosphorylation.

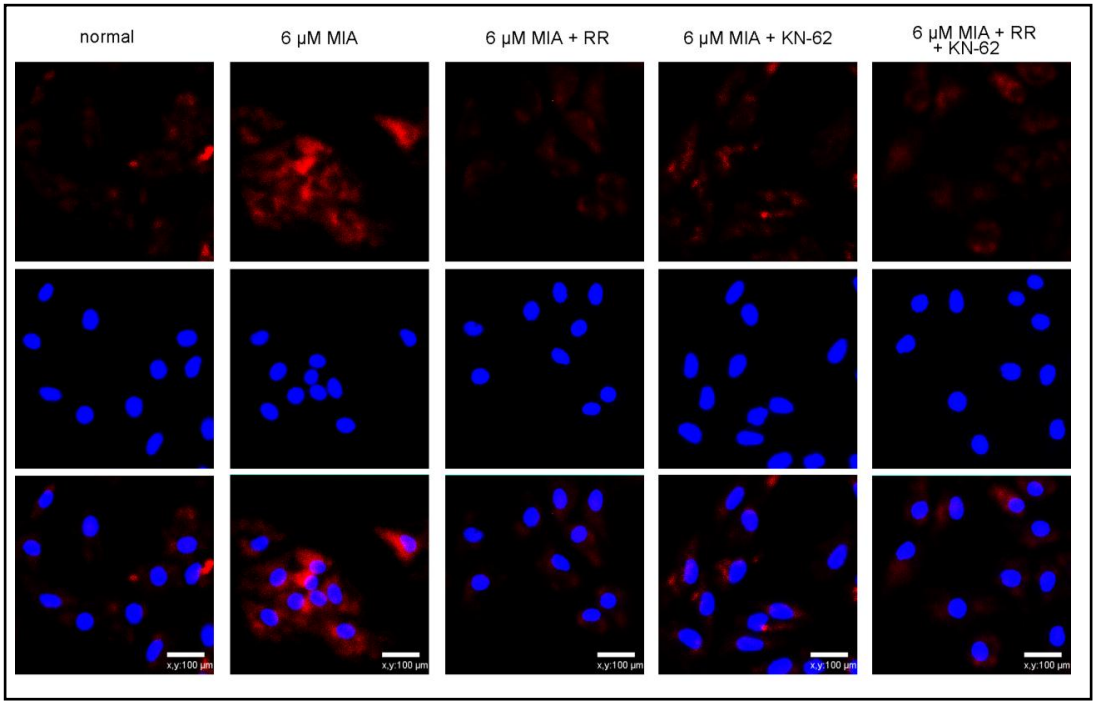

Fig. 6. P-CAMKII expression in different chondrocyte treatment groups. Expression of (A) p-CAMKII (red) was determined by immunofluorescence staining and blue staining indicates the nucleus (original magnification ×200). MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; p-CAMK, Ca2+/Calmodulin-Dependent Kinase II phosphorylation.

the fluorescence intensity did not decrease. A graph of the fluorescence intensity of calcium ions in each group is shown in Fig. 5B. This indicates that the increased calcium influx in response to treatment with MIA may be inhibited by the inhibition of TRPV5, but not by the inhibition of CaMKII phosphorylation. These results indicate that TRPV5 has a specific role in mediating extracellular Ca^{2+} influx in OA.

Primary chondrocytes were stained with anti-collagen II as an indication of functional chondrocytes. We observed p-CAMKII expression in chondrocytes *in vitro* by immunofluorescence staining. As shown in Fig. 6, p-CAMKII protein was aggregated into large clumps in the perinuclear areas of chondrocytes. The fluorescence intensity for cells of the 6 μM MIA alone group was obviously stronger than for cells of the 0 μM MIA group. However, the fluorescence intensity was reduced markedly by treating with 10 μM ruthenium red and 10 μM KN-62. Treatment with 10 μM ruthenium red combined with 10 μM KN-62 showed the same inhibition as treatment with 10 μM ruthenium red or 10 μM KN-62 alone. The two experiments above indicated that p-CAMKII activation required calcium influx that was mediated by TRPV5, which was suppressed by ruthenium red. Also, CAMKII phosphorylation activated by calcium influx was abolished by KN62.

CAMKII phosphorylation is activated by an increase of calcium mediated by TRPV5 in chondrocytes, which then initiates chondrocyte apoptosis

We used flow cytometry to study apoptosis in chondrocytes. As shown in Fig. 7, flow cytometric analysis revealed that the percentage of apoptotic cells significantly increased for chondrocytes from the 6 μM MIA group of rats compared with chondrocytes from control group (0 μM MIA) rats. This suggests that MIA stimulation can lead to chondrocyte apoptosis which simulates the degeneration of articular cartilage in OA *in vitro*. However, the percentage of apoptotic cells was dramatically attenuated in the presence of ruthenium red. Furthermore, the inhibition of CaMKII phosphorylation with KN-62 also markedly attenuated the percentage of apoptotic cells. Consistently, an attenuation of the percentage of apoptotic

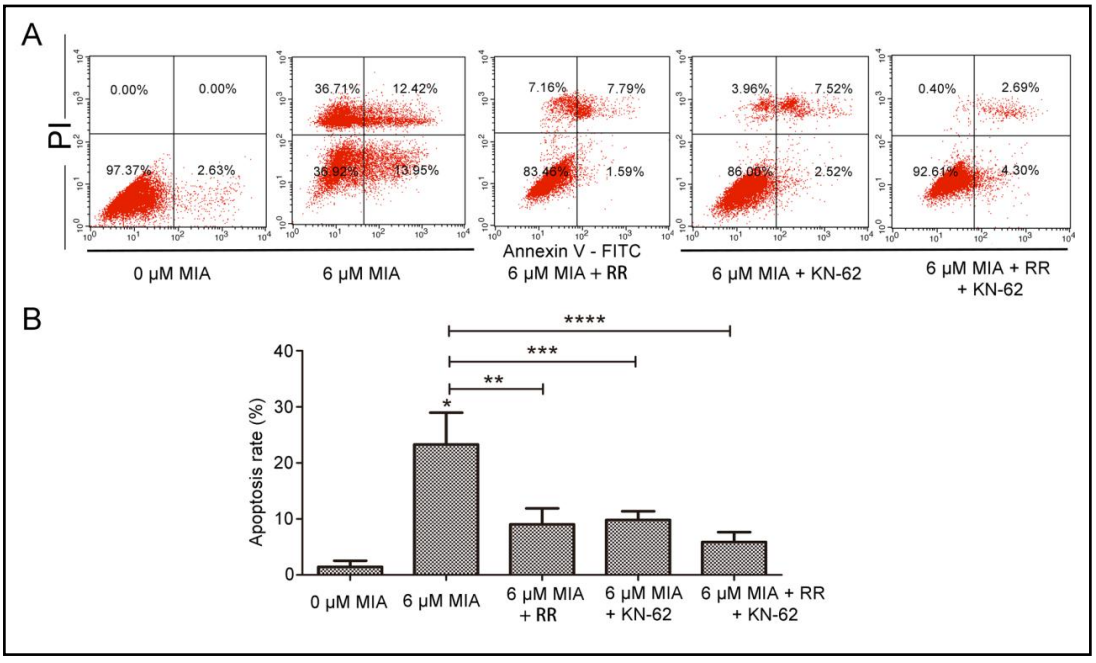

Fig. 7. Each group chondrocyte apoptosis detected by flow cytometry with Annexin V-FITC/PI staining of chondrocytes (A) Each group apoptotic chondrocytess distribution in flow cytometry machine. (B) Bar graph showing the apoptosis rate of each group. *P<0.05 difference vs. untreated group (control); #P<0.05; **P<0.05, ***P<0.05, ****P<0.05 difference vs. 6 μM MIA without therapy treatment. Each column represents mean ± SEM (n = 3). MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine.

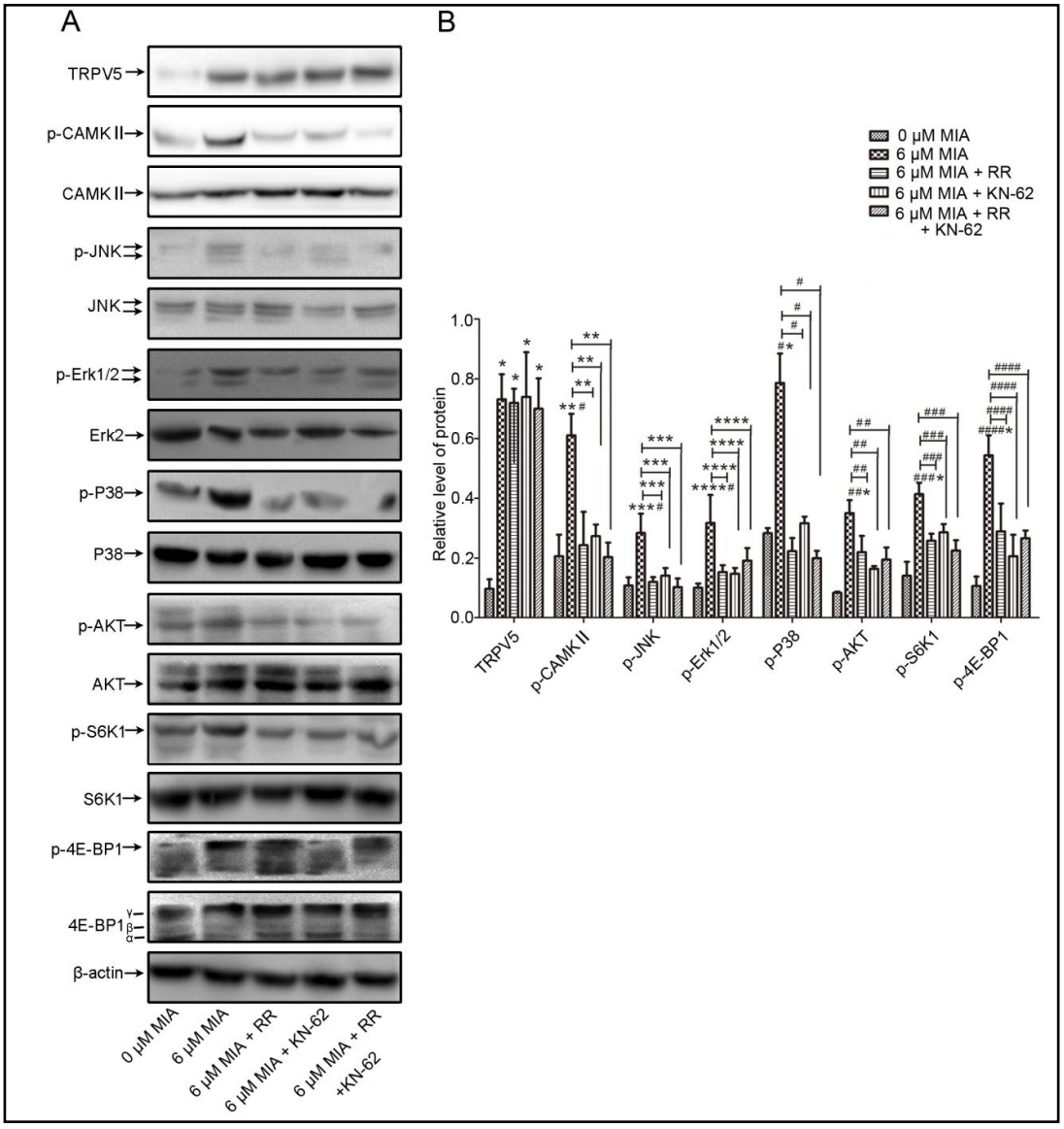

Fig. 8. Ca^{2+} mediated through TRPV5 elicited CaMKII phosphorylation activation leading to chondrocyte apoptosis by activating the MAPK and Akt/mTOR pathways (A) TRPV5, p-CAMKII, p-JNK, p-Erk, p-38, p-Akt, p-S6k1, p-4E-BP1 protein expression from groups were detected by western blotting. (B) A bar graph showing relative levels of TRPV5, p-CAMKII, p-JNK, p-Erk, p-38, p-Akt, p-S6k1, p-4E-BP1 proteins. *P<0.05; **P<0.05; ***P<0.05; ****P<0.05; *****P <0.05; #P<0.05; ###P<0.05; ####P<0.05 #####P<0.05 vs. untreated control. **P<0.05; ***P<0.05; ****P<0.05; #P <0.05; ##P<0.05; ###P<0.05; ####P<0.05 vs. 6 μM MIA without therapy treatment. MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; TRPV5, transient receptor potential channel vanilloid 5; p-CAMKII, Ca2+/calmodulin-dependent kinase II phosphorylation; MAPK, mitogen-activated protein kinase; p-JNK, c-Jun N-terminal kinase phosphorylation; p-Erk, extracellular signal-regulated protein kinase phosphorylation; p-Akt, protein kinase B phosphorylation; 4E-BP1, 4E-binding protein; p-S6k1, S6 kinase 1 phosphorylation.

cells effect could also be detected for treatment with 10 μM ruthenium red combined with 10 μM KN-62. A graph of the percentage of apoptotic cells in each group is shown in Fig. 7B and is consistent with results shown in Fig. 7A. These results indicate that the activation of p-CAMKII requires calcium influx mediated by TRPV5, which is essential for chondrocyte apoptosis. Apoptosis in chondrocytes can be diminished and even reversed by ruthenium red and KN62, indicating apoptosis was mediated by TRPV5 and CaMKII phosphorylation.

Ca^{2+} influx mediated by TRPV5 elicited CaMKII phosphorylation and led to chondrocyte apoptosis by activating MAPK and mTOR pathways

We sought to determine whether Ca^{2+}, mediated by the TRPV5-mediated induction of CaMKII phosphorylation, correlated with its activation of MAPK and mTOR signaling pathways. The expression of core proteins was determined by western blotting. As shown in Fig. 8A, chondrocytes were exposed to MIA (6 μM) and pretreated with ruthenium red, KN-93 or both ruthenium red plus KN-93. We found that TRPV5 was markedly upregulated after the stimulation of cells with 6 μM MIA. The phosphorylation of CaMKII was also markedly upregulated with 6 μM MIA stimulation, but was attenuated by pretreatment with ruthenium red, KN-62 or ruthenium red plus KN-62. The phosphorylation of Erk1/2, JNK, and p38 MAPK in chondrocytes showed similar changes as for p-CaMKII protein. But Erk1/2, JNK, and p38 MAPK total proteins did not show a change. The phosphorylation of CaMKII activates MAPK signaling pathways by phosphorylating cascade proteins in the MAPK pathway; in chondrocytes, these showed a similar change as for p-CaMKII protein. We also found that the phosphorylation of Akt, S6K1, and 4E-BP1 in chondrocytes showed a similar change as p-CaMKII protein, simultaneously. But AKT, S6K1, and 4E-BP1 total proteins did not show any change. The activation of Akt, S6K1, and 4E-BP1 was inhibited markedly by ruthenium red and KN-62. These results indicate that phosphorylation of CaMKII activates Akt/mTOR signaling pathways by phosphorylating cascade proteins in the Akt/mTOR pathway. A graph of relative protein levels in repeated experiments is shown in Fig. 4B. The results indicate that TRPV5-mediated Ca^{2+} influx elicited chondrocyte apoptosis by inducing CaMKII phosphorylation that then activated the MAPK and Akt/mTOR pathways.

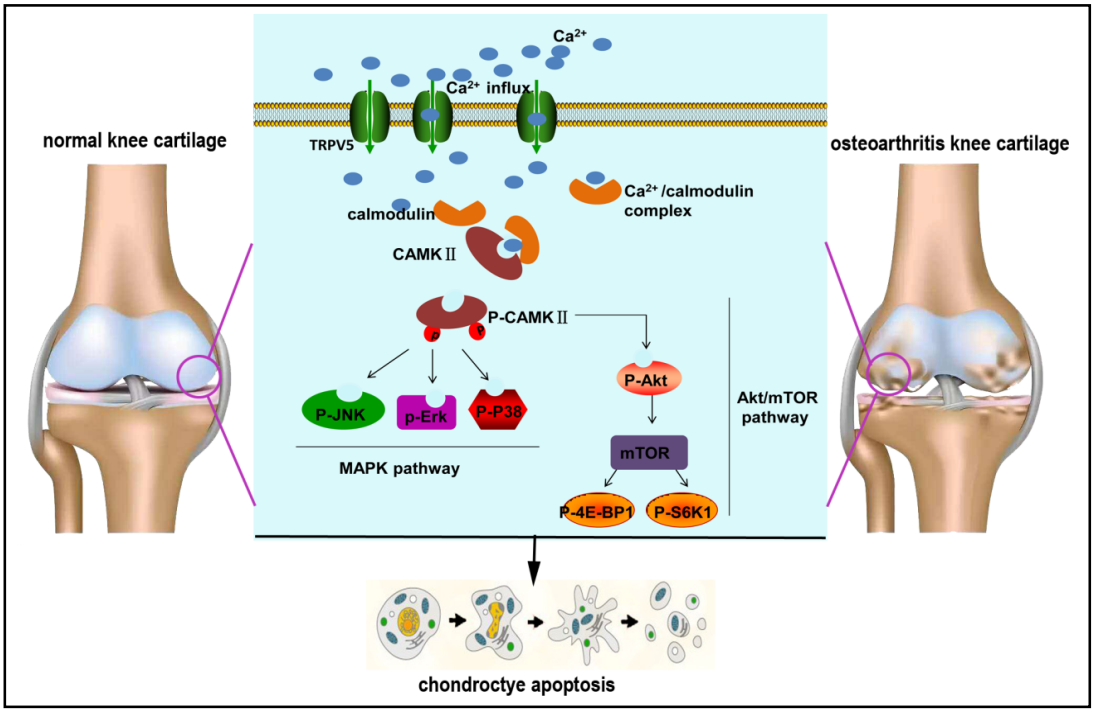

Fig. 9. Diagram of the signaling cascade The up-regulated TRPV5 could be an initiating factor that activate CaMKII phosphorylation via the mediation of Ca2+ influx. Activated p- CaMKII play a critical role in contributing to chondeocyte apoptosis via MAPK and Akt/mTOR pathways. MIA, monosodium iodoacetate; RR, ruthenium red; KN-62, 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; TRPV5, transient receptor potential channel vanilloid 5; p-CAMKII, Ca2+/calmodulin-dependent kinase II phosphorylation; MAPK, mitogen-activated protein kinase; p-JNK, c-Jun N-terminal kinase phosphorylation; p-Erk, extracellular signal-regulated protein kinase phosphorylation; p-Akt, protein kinase B phosphorylation; mTOR, mammalian target of rapamycin; 4E-BP1, 4E-binding protein; p-S6k1, S6 kinase 1 phosphorylation.

Discussion

Based on current knowledge, chondrocyte apoptosis may be the underlying factor for the initiation of OA [3]. Therefore, understanding the mechanism by which chondrocytes undergo apoptosis is essential for developing appropriate targeted therapies for OA treatment. Considering the ethical problems of human experimentation and individual variability, in order to study the mechanism of chondrocyte apoptosis, we sought to establish a stable animal model of osteoarthritis. Therefore, an MIA-induced experimental OA rat model was developed to imitate the degeneration of articular cartilage observed in human disease. The MIA-induced experimental OA rat model has been widely used to study OA pathogenesis [16, 17]. The advantages of such a model are that it involves a quick and easy procedure, produces OA-like lesions, and displays functional impairment similar to that observed in human disease [18].

Since the TRPV family was first discovered in early 1997 [19] and was systematically proposed in 2001 [20], TRPV proteins have been investigated in the etiologies of many diseases. TRPV5 is a member of the TRPV subfamily that functions as a facilitative Ca^{2+} transporter. Our study delineated that Ca^{2+} increases via intracellular influx through TRPV5 can inhibit chondrocyte autophagy in OA [7]. In this study, we have also comprehensively demonstrated TRPV5 expression in cartilage and that the upregulation of TRPV5 participates in the development of OA in the MIA-induced rat model. We also found p-CaMKII was significantly upregulated in OA cartilage in a positive linear relationship with TRPV5 protein (Fig. 3, Fig. 4). In this study, the inhibition of TRPV5 using ruthenium red had a protective effect against the development of OA equal to the inhibition of CaMKII phosphorylation. We speculate that the activation of p-CaMKII associated with OA may be promoted by TRPV5-mediated Ca^{2+} influx. It is worth mentioning that ruthenium red is a non selective calcium channel blocker. It interacts with not only TRPV5 channels, but also the ryanodine receptor (RyR) family [21] and other TRPV family [22] of ion channels; ruthenium red even interacts with the mitochondrial Ca^{2+} uniporter (MCU) [23]. Therefore, when we utilized ruthenium red to inhibit TRPV5 ion channels, other calcium channels may also have been inhibited by ruthenium red because of its non-selectivity. However, our previous results [24] showed that intracellular calcium ions were significantly reduced by adding ruthenium red and TRPV5 knock-down in chondrocytes, and these reduction effects were roughly the same. Therefore, we speculate that the primary effect of ruthenium red was to act as an inhibitor of upregulated TRPV5, then inhibit extracellular calcium influx in chondrocytes. The reason may be that other calcium channels have no obvious obstructed effect due to other calcium channels expression level might have not changed or at least not be confirmed now in osteoarthritis chondrocytes.

It has been previously reported that abnormal TRPV5 can cause Ca^{2+} influx overload in HEK293 [25] and mice ear hair cells [26]. Recently, our study reported that a Ca^{2+} increase via intracellular influx through TRPV5 can inhibit chondrocyte autophagy in OA [7]. Consistent with that study, here we noted that upregulated TRPV5 was able to increase the elevation of Ca^{2+} influx in chondrocytes. Calcium ions, as second messengers, mediate a variety of physiological responses in cells and have direct and indirect roles in mediating apoptosis [4]. Ca^{2+} was shown to be important in apoptosis as it is involved the production of calcium entry-dependent reactive oxygen species (ROS) [27], mitochondrial depolarization, and DNA fragmentation [28]. Although most studies regarding the role of Ca^{2+} in apoptosis have focused on its increased release from the endoplasmic reticulum, the role of Ca^{2+} influx through TRP channels has also been demonstrated recently [29]. Our results show that calcium influx though the TRPV5 channel can induce the apoptosis of chondrocytes, which can be diminished and even reversed by ruthenium red and KN62 (Fig. 5) [30]. We speculate that the activation of p-CaMKII promoted by TRPV5-mediated Ca^{2+} influx may be a core event in the induction of chondrocyte apoptosis.

CaMKII, a multifunctional Ser/Thr kinase ubiquitously expressed in chondrocytes [31], is involved in numerous Ca^{2+}-sensitive processes [32]. It is activated upon binding of

CaM, which undergoes autophosphorylation. Based on the unique regulatory properties of CaMKII and our recent findings that TRPV5 induces Ca^{2+} influx contributing to chondrocyte apoptosis, we speculate that CaMKII is an "interpreter" of the TRPV5 induction of Ca^{2+} signaling, leading to apoptosis in chondrocytes. In the present study, we observed that exposure of chondrocytes to MIA resulted in p-CaMKII (Fig. 6), which was consistent with an increased rate of apoptosis. If intracellular Ca^{2+} influx is inhibited with ruthenium red or the activation of CaMKII blocked, chondrocyte apoptosis can be attenuated and even reversed (Fig. 7).

Increasing evidence indicates that p-CaMKII activates the MAPK cascade [33]. Consistent with this hypothesis, in this study, we noted that phosphorylation of Erk1/2, JNK, and p38 MAPK in chondrocytes all increased under p-CaMKII activation. However, the phosphorylation of Erk1/2, JNK, and p38 MAPK was abolished when the phosphorylation of CAMKII was inhibited by KN-62 (Fig. 8). Here, we also noticed that the elevation of p-CAMKII did not alter the total cellular protein expression of JNK1/2, but induced both phosphorylation of JNK2 (the upper band) and JNK1 (the lower band). However, in contrast, p-CAMKII preferentially induced p-JNK1 in neurons [34]. We speculate both p-JNK2 and p-JNK1 are critical to the phosphorylation of c-Jun. Four isoforms of p38 (-α, -β, -γ, and -δ) have been identified in chondrocytes in OA [35]. In this study, an antibody to phospho-p38 (Thr180/Tyr182; Cat. #9215, Cell Signaling) was used that could not differentiate the -α, -β, -γ, and –δ isoforms of p38. Therefore, currently, we do not know what isoforms of p38 MAPK are activated by p-CaMKII. Since various isoforms of p38 have unique cellular functions, this suggests that the identification of the exact isoforms of p38 in OA is important.

It is commonly accepted that mTOR is a master kinase, which positively regulates protein synthesis, cell growth, proliferation, and survival [36]. Akt/mTOR signaling is crucial for chondrocyte survival [37]. We have demonstrated that p-CaMKII activates the Akt/mTOR signaling pathway, promoting chondrocyte apoptosis. We found that p-CaMKII activated the phosphorylation of Akt, S6K1, and 4E-BP1 in chondrocytes under 6 μM MIA stimulation. As expected, we found that pre-treatment with KN-62 or ruthenium red markedly attenuated Cd-induced phosphorylation of Akt, S6K1, and 4E-BP1, as well as chondrocyte apoptosis (Fig. 8). In contrast, several studies have shown that blocking the Akt/mTOR pathway suppresses proliferation and promotes apoptosis in many kinds of tumors [38, 39]. As mTOR controls cap-dependent translation [36], we hypothesize a possible mechanism whereby the activation of mTOR increases protein synthesis, which may then consume a lot of energy (ATP) and generate high levels of ROS. If mTOR is activated continuously, ATP would become exhausted, leading to apoptosis.

Conclusion

In summary, we found that the TRPV5 cation channel is functionally upregulated in OA articular cartilage. Upregulated TRPV5 may be an initiating factor that activates CaMKII phosphorylation via the mediation of Ca2+ influx. Activated p-CaMKII plays a critical role in contributing to chondrocyte apoptosis via MAPK and Akt/mTOR pathways (Fig. 9). Our results underscore an intriguing role for TRPV5 and p-CaMKII as mediators and potential drug targets in OA.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (General Program; No.: 81272050; No.: 81772420); Excellent PhD Program of ShengJing Hospital of China Medical University (No.: ME332).

Disclosure Statement

The authors declare no conflicts of interest.

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