International Immunopharmacology 66 (2019) 347–353

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Ivabradine abrogates TNF-α-induced degradation of articular cartilage matrix

Xianxiang Xiang[a,b,1], Yantao Zhou[c,1], Honglin Sun[d], Shiping Tan[e], Zhanbin Lu[e], Lixin Huang[a,*], Weiming Wang[f,**]

[a] Department of orthopedics, The First Affiliated Hospital of Soochow University, Suzhou 215000, China
[b] Dalian Jiuzhou Century Hospital, Dalian 116400, China
[c] Department of Orthopedics, The central Hospital of Enshi Autonomous Prefecture, Enshi 445000, China
[d] Department of Internal medicine, Affiliated Zhongshan Hospital of Dalian University, Dalian 116001, China
[e] Department of orthopedics, Dalian Jiuzhou Century Hospital, Dalian 116400, China
[f] Department of orthopedics, Affiliated Zhongshan Hospital of Dalian University, Dalian 116001, China

ARTICLE INFO

Keywords:
Ivabradine
Osteoarthritis
Extracellular matrix
Type II collagen
Aggrecan

ABSTRACT

Ivabradine is most commonly used for the treatment of worsening cardiac failure in patients who cannot tolerate the maximum dose of β-blockers or in whom treatment with β-blockers is contraindicated. While ivabradine is regarded as a highly selective "funny current" (I_f) inhibitor, the molecular mechanism behind the effect of this drug remains poorly understood. In the present study, we applied ivabradine in the context of osteoarthritis by treating primary human chondrocytes with tumor necrosis factor-α (TNF-α) and measuring degradation of the articular cartilage matrix as well as the expression of various enzymes and pro-inflammatory cytokines. Our results indicate that ivabradine significantly abrogated TNF-α-induced up-regulation of matrix metalloproteinase-3 (MMP-3), MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4, and ADAMTS-5 at both the gene and protein levels. Notably, ivabradine attenuated TNF-α-induced reduction of type II collagen and aggrecan at both the mRNA and protein levels. Also, we found that ivabradine inhibited the expression and secretion of interleukin-6 (IL-6) and interleukin-1β (IL-1β) as well as the production of reactive oxygen species (ROS). Mechanistically, our results indicate that ivabradine abolished the activation of nuclear factor (NF-κB) by inhibiting nuclear translocation of NF-κB p65. Knockdown of HCN2 enhanced the protective effects of ivabradine against TNF-α- induced degradation of both type II collagen and aggrecan, suggesting that the inhibitory effects of ivabradine in ECM degradation might be mediated by HCN2. Our findings demonstrate that ivabradine may indeed have a potential application in preventing excessive degradation of the articular cartilage matrix, thereby preventing the pathological development and progression of osteoarthritis.

1. Introduction

Ivabradine is an antiarrhythmic medication marketed by Amgen under the name of Corlanor in the U.S. It was approved by the European Food and Drug Administration (FDA) in 2005 and the United States FDA in 2015. As a hyperpolarization-activated cyclic nucleotide-gated (HCN) channel blocker, ivabradine specifically inhibits the so-called "funny current" (I_f) involved in pacemaker activity. Ivabradine is mainly prescribed for the treatment of worsening chronic heart failure in patients already prescribed the maximum tolerated dose of β-blockers or in whom the use of β-blockers is contraindicated. Ivabradine treatment has been shown to reduce hospitalization due to cardiac symptoms and the risk of mortality by 20–30%, with the most common side effects being bradycardia, hypertension, atrial fibrillation and luminous phenomena, all of which occurred in ≤10% of patients. The normal starting dose of ivabradine is 5 mg twice daily, with the maximum dose being 7.5 mg twice daily [1, 2]. The molecular structure of ivabradine is shown in Fig. 1. However, the exact molecular

* Correspondence to: L. Huang, Department of orthopedics, The First Affiliated Hospital of Soochow University, No. 899, Pinghai Road, Suzhou 215000, Jiangsu, China.
** Correspondence to: W. Wang, Department of orthopedics, Affiliated Zhongshan Hospital of Dalian University, Dalian, 116001, China, No. 6, Jiefang Street, Zhongshan District, Dalian 116001, Liaoning, China.
E-mail addresses: szhuanglx@yeah.net (L. Huang), oldpal@126.com (W. Wang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.intimp.2018.11.035
Received 19 September 2018; Received in revised form 8 November 2018; Accepted 20 November 2018
Available online 03 December 2018

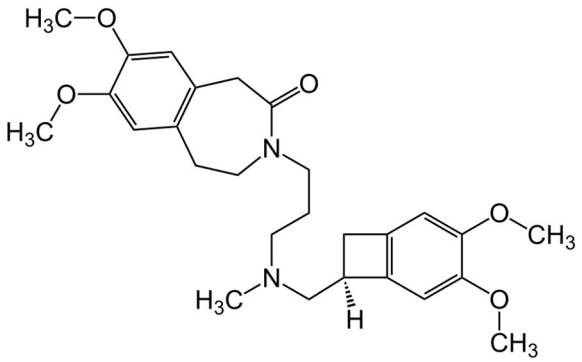

Fig. 1. Molecular structure of ivabradine.

mechanism remains poorly understood. In the past decades, multiple pharmacological capacity of ivabradine has been reported. For example, ivabradine has been reported to exert a protective role in cardiac function in streptozotocin (STZ)-induced diabetic mice by inhibiting JNK/p38 MAPK-mediated inflammation and apoptosis [3]. Notably, ivabradine is able to protect endothelial cells against low shear stress (LSS)-induced inflammation and oxidative stress [4].

Osteoarthritis (OA) is a global health problem most prevalent among the elderly. Excessive degradation of the articular extracellular matrix (ECM) is one of the hallmarks of the onset and pathological progression of OA. The main components of the articular ECM are type II collagen and aggrecan. Type II collagen is turned over at a very slow rate, so excessive degradation of type II collagen is largely considered to be irreversible [5]. As the main structural support of the ECM, type II collagen provides the rigidity necessary to withstand mechanical forces, while aggrecan gives the ECM its shock-absorptive property [6]. In the pathology of OA, metalloproteinases (MMPs) and especially MMP-3 and MMP-13 are regarded as collagenases which exert enzymatic break down of type II collagen. Upregulation of MMP expression is recognized as an early event in the development of OA [7]. Similarly, the aggrecanases a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5 degrade aggrecan, furthering breakdown of the ECM and contributing to the pathological nature of OA [8]. Other contributing factors are generation of reactive oxygen species (ROS) and proinflammatory cytokines such as interleukin (IL)-6 and IL-1β which promote and sustain the inflammatory response, thereby contributing to the chronic nature of the disease [9]. Additionally, activation of NF-κB has been shown to be elevated in patients with OA [10].

In the present study, for the first time to our knowledge, we investigated the effects of ivabradine on human primary chondrocytes (HPCs) to explore its potential in the treatment of OA. As a relatively new and rarely prescribed medication, there is great potential for uses of ivabradine outside of cardiac indications. Considering this, we exposed HPCs to 10 ng/ml TNF-α for 24 h and found that treatment with 20 or 50 nM ivabradine could successfully ameliorate degradation of type II collagen and aggrecan; downregulate expression of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5; mitigate production of ROS, IL-6 and IL-1β; and prevent nuclear translocation of p65 and subsequent activation of NF-κB in HPCs in vitro.

2. Materials and methods

2.1. Cell culture

Experiments with human primary chondrocytes (HPCs) were performed as previously descried [11] and in compliance with human subject protocols reviewed and approved by the institutional review board in our institute (No. 20150023). All participants in the study signed written informed consent. Isolated HPCs were cultured in Dulbecco's modified Eagle's high glucose medium (DMEM) with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 IU/ml penicillin in a humid 37 °C incubator. Once the cultured primary chondrocytes had reached confluence, they were either collected and stored at −80 °C for later analyses, or re-plated at 3×10^5 cells per well in 12-well plates or 7×10^5 cells per well in 6-well plates for all other experiments. Ivabradine was from Sigma-Aldrich (#SML0281), USA. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. To knock down the expression of HCN2, cells were transducted with HCN2 shRNA lentivector (#i009495, Applied biological materials, CANADA) in accordance with the manufacturer's instructions.

2.2. Quantitative real-time PCR analysis

Total RNA was isolated using Qiazol lysis reagent (Qiagen, USA) in accordance with the manufacturer's instructions. Nuclear RNA was isolated using a nuclear extraction reagent from Thermo Fisher Scientific (Cat# 78833). The concentrations of RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). To evaluate mRNA expression, 2 μg of total RNA was reverse-transcribed into cDNA using oligo dT primers. Real-time PCR was performed on a Roche LightCycler 480 (Roche Applied Science, Switzerland) using a 96-well or 384-multiwell format. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Gene expression is presented as $2^{-\Delta\Delta Ct}$ with respect to non-stimulated cells. MMP-3 (forward: 5′-TTAAAATAAAACTGCTTTT-3′ and reverse: 5′-AACTGGAGCATTTTTT-3′); MMP-13 (forward: 5′-AGG AGC ATG GCG ACT TCT AC-3′; reverse: 5′-TAA AAA CAG CTC CGC ATC AA-3′); ADAMTS-4 (forward: 5′-ACACTGAGGACTGCCCAAC-3′; reverse: 5′-GGTGAGTTTGCACTGGTCCT-3′); ADAMTS-5 (forward: 5′-GCAGAA CATCGACCAACTCTACTC-3′; reverse: 5-CCAGCAATGCCCACCG AAC-3′); IL-1β (forward: 5′-TACCTGTCCTGCGTGTTGAA-3′; reverse: 5′-TCTTTGGGTAATTTTTGGGATCT-3′); IL-6 (forward: 5′-TTGGGAAG GTTACATCAGATC-3′; reverse: 5′-GGGTTGGTCCATGTCAATTT-3′); GAPDH (forward: 5′-ACT GGC GTC TTC ACC ACC AT-3′; reverse: 5′-AAG GCC ATG CCA GTG AGC TT-3′).

2.3. Protein purification and Western blot analysis

Cells were harvested after treatment and then washed with ice-cold PBS and homogenized in ice-cold RIPA lysis and extraction buffer (Cat# 89900, Invitrogen) supplemented with protease inhibitor (EDTA-free protease mixture inhibitor) and phosphatase inhibitor (Roche Diagnostics, Netherlands) by incubating on ice for 15 min. Cells were then collected into 1.5 ml tubes. The homogenates were centrifuged at 12,000 × *g* for 10 min and the supernatant was used for further analysis. Protein concentration was determined using the Micro BCA™ protein assay kit (Cat# 23235, Thermo Fisher Scientific, USA). Equal amounts of proteins (20 μg/lane) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, USA). Blots were blocked with 5% skim milk for 1 h and then incubated with primary antibodies overnight in a cold room on a shaker. On the second day, after several washes in TBS-T, blots were incubated with secondary antibodies conjugated with HRP for 2 h. Immunoreactivity was visualized using the ECL PLUS Western blotting detection reagent (GE Healthcare, USA). For quantitative analysis of the blots and in-situ micrographs, the band intensities were measured densitometrically using Image J software (U.S. National Institutes of Health, USA). The following antibodies were used in this study: mouse monoclonal antibody (mAb) against type II collagen (1:1000, #MAB8887, Chemicon, USA); mouse mAb against aggrecan (1:1000, #ab3778, Abcam, USA); rabbit polyclonal antibody (pAb) against MMP-3 (1:2000, #ab53015, Abcam, USA); rabbit polyclonal antibody (pAb) against MMP-13 (1:2000, #ab84594, Abcam, USA);

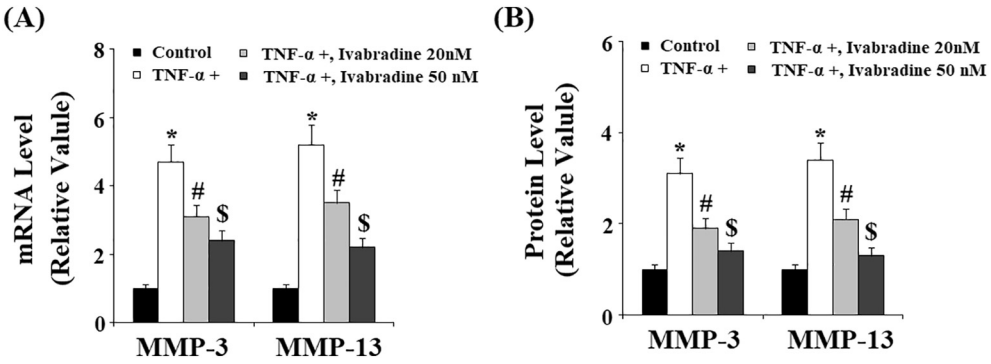

Fig. 2. Ivabradine abrogated up-regulation of MMPs induced by TNF-α in human primary chondrocytes. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. (A). mRNA levels of MMP-3 and MMP-13 were determined by real-time PCR; (B). Protein levels of MMP-3 and MMP-13 were determined by ELISA (*, #, $, $P < 0.01$ vs. previous group).

rabbit pAb against ADAMTS-4 (1:2000, #ab185722, Abcam, USA), rabbit pAb against ADAMTS-5 (1:2000, #ab41037, Abcam, USA); rabbit monoclonal antibody against p65 (1: 5000, #4767, Cell Signaling Technology, USA), mouse monoclonal antibody against Lamin B (1: 5000, sc-374015, Cell Signaling Technology, USA); mouse mAb against β-actin (1:10000, #3700, Cell Signaling Technology, USA); Anti-rabbit IgG, HRP-linked Antibody (1:2000, #7074, Cell Signaling Technology, USA); Anti-mouse IgG, HRP-linked Antibody (1:2000, #7076, Cell Signaling Technology, USA).

2.4. ELISA assay analysis

Protein expressions of MMP-3, MMP-13, ADAMTS-4, ADAMTS-5, IL-6, and IL-1β were determined using an enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions: MMP-3 (#DMP300, R&D systems); MMP-13 (#DY511, R&D systems); ADAMTS-4 (#DY4307-05, R&D systems); ADAMTS-5 (#DY2198-05, R &D systems); IL-6 (#D6050, R&D systems); IL-1β (#DLB50, R&D systems). Briefly, 96-well plates were coated with antibodies against MMP-3, MMP-13, ADAMTS-4, ADAMTS-5, IL-6, IL-1β and incubated overnight at 4 °C. Plates were then blocked with goat serum at RT for 1 h. Samples were added to each well and incubated for 3 h at RT. After 3 washes with washing buffer, biotinylated sheep polyclonal antibodies were added and incubated at RT for 1 h. The plates were then washed 3 times and incubated with 50 μl of avidin-HRP (diluted 1:5000). The reaction was stopped with H_2SO_4. Absorbance was recorded at 490 nm.

2.5. Determination of intracellular reactive oxygen species (ROS)

Intracellular ROS in human primary chondrocytes were examined using a 2,7-dichlorofuorescin diacetate (DCFH-DA) staining assay. After the necessary treatment, cells were loaded with 1 μM DCFH-DA (Sigma-Aldrich, USA) in culture medium without FBS for 15 min in darkness. After 3 washes, fluorescent signals were recorded by a DM500 fluorescence microscope (Leica Microsystems, Germany). ROS quantification was performed using the Image J software. Briefly, regions of interest (ROI) were defined and the average number of cells present in the previously defined ROI was determined. The integrated density value (IDV) in ROI was assessed. The IDV was divided by the average number of cells and was used to index average level of intracellular ROS.

2.6. NF-κB promoter-luciferase assay

The patterns of NF-κB were determined using NF-κB promoter-luciferase (Clontech, USA) and β-galactosidase plasmids. Plasmids were transfected into chondrocytes using Lipofectamine 2000 following the manufacturer's instructions. Cells were treated with ivabradine at concentrations of 20 and 50 nM for 24 h followed by treatment with TNF-α (10 ng/ml) for another 24 h. Cell lysates were collected and used for measuring luciferase activity and β-galactosidase activity using a Secrete-Pair™ Dual luminescence assay kit (Gene Copoeia, MD) on a luminometer (Infinite F500 Multimode Reader). Luciferase activity was normalized to β-galactosidase activity.

2.7. Preparation of nuclear extracts

To determine the nuclear translocation of p65, nuclear protein was isolated from chondrocytes using a nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, USA). Briefly, cells were lysed in a hypotonic buffer on ice for 15 min and centrifuged at 500 ×*g* for 1 min to pellet nuclei. Pellets were then re-suspended in nuclear extract buffer and incubated for 15 min on ice followed by centrifugation at 14,000 ×*g* for 10 min. Supernatants were collected for Western blot analysis.

2.8. Statistical analysis

Statistical analyses were performed using SPSS Statistics software (version 19.0). Comparisons between multiple groups were done using ANOVA followed by Bonferroni post-hoc test. A value of $P < 0.05$ was assumed to indicate a significant difference.

3. Results

3.1. Ivabradine abrogated TNF-α-induced upregulation of MMPs

Production of MMPs is recognized as a critical event in the development and progression of osteoarthritis as well as other chronic inflammatory diseases. To investigate the effects of ivabradine on the upregulation of MMPs induced by TNF-α, we exposed cultured HPCs to 10 ng/ml TNF-α in the presence or absence of 20 or 50 nM ivabradine for 24 h. The results of real-time PCR and ELISA analysis show that ivabradine significantly reduced the increased expression of MMPs induced by TNF-α at both the mRNA and protein levels in a dose-dependent manner (Fig. 2).

3.2. Ivabradine mitigated TNF-α-induced degradation of type II collagen

Reduced synthesis and excessive degradation of type II collagen are irreversible events that contribute to the chronic and debilitating nature of OA. Briefly, we exposed HPCs to 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. As shown in Fig. 3A, the results of real time PCR analysis indicate that TNF-α reduced the expression of type II collagen at the mRNA level, which was prevented by ivabradine in a dose-dependent manner. Consistently, the results of Western blot analysis in Fig. 3B reveal that ivabradine mitigated degradation of type II collagen induced by TNF-α in a dose-dependent manner, which is consistent with our previous finding that ivabradine ameliorated the upregulation of MMPs induced by TNF-α.

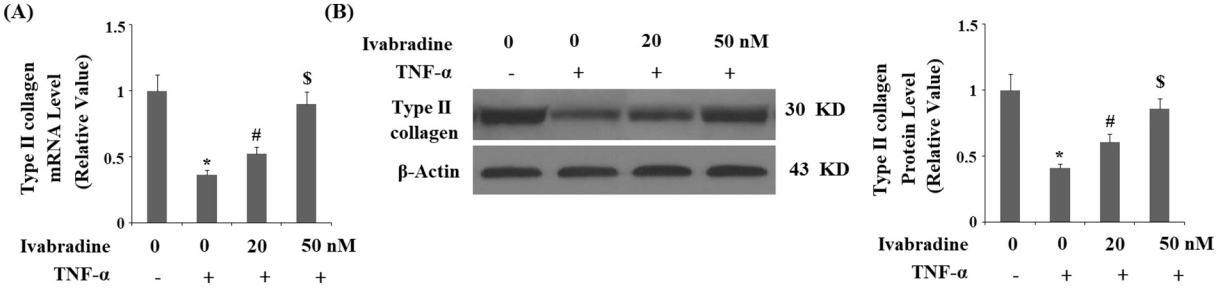

Fig. 3. Ivabradine mitigated TNF-α-induced degradation of type II collagen in human primary chondrocytes. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. Expression of type II collagen was determined by Western blot analysis (*, #, $, $P < 0.01$ vs. previous column group).

3.3. Ivabradine abrogated TNF-α-induced expression of ADAMTS-4 and ADAMTS-5

Expression of ADAMTS-4 and ADAMTS-5 induced by TNF-α is an important event in the initiation of degradation of the articular ECM. To investigate the effects of ivabradine on the expression of ADAMTS-4 and ADAMTS-5 induced by TNF-α, we exposed HPCs to 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. As demonstrated by the results of real-time PCR and ELISA analyses in Fig. 4, ivabradine successfully abrogated the expression of ADAMTS-4 and ADAMTS-5 induced by TNF-α at both the mRNA and protein levels in a dose-dependent manner.

3.4. Ivabradine mitigated TNF-α-induced degradation of aggrecan in HPCs

As a main component of the articular ECM, degradation of aggrecan induced by TNF-α is another major event in the development and progression of OA. To investigate the effects of ivabradine treatment on the expression of aggrecan in the articular ECM, we exposed HPCs to 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. As shown in Fig. 5A, the results of real time PCR analysis indicate that TNF-α reduced the expression of aggrecan at the mRNA level, which was prevented by ivabradine in a dose-dependent manner. Concordant with our previous finding that ivabradine abrogated expression of the aggrecanases ADAMTS-4 and ADAMTS-5, the results of Western blot analysis shown in Fig. 5B indicate that treatment with ivabradine significantly ameliorated degradation of aggrecan in a dose-dependent manner.

3.5. Ivabradine mitigated TNF-α-induced production of IL-6 and IL-1β

The inflammatory cytokines IL-6 and IL-1β play a major role in a variety of chronic inflammatory conditions including OA. To determine the effect of ivabradine on the production of IL-6 and IL-1β induced by TNF-α in chondrocytes, we exposed HPCs to 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. The results of real-time PCR and ELISA analyses indicate that treatment with ivabradine mitigated the production of IL-6 and IL-1β induced by TNF-α in a dose-dependent manner (Fig. 6).

3.6. Ivabradine mitigated TNF-α-induced generation of ROS

Generation of ROS has been widely shown to play a critical role in sustaining chronic inflammatory and degradative effects in OA. We investigated the effect of ivabradine on generation of ROS by exposing HPCs to 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. As shown in Fig. 7, the results of DCFH-DA assay reveal that ivabradine significantly reduced TNF-α-induced generation of ROS in a dose-dependent manner.

3.7. Ivabradine mitigated TNF-α-induced activation of NF-κB

Activation of NF-κB is a well-known event in the initiation and progression of numerous chronic inflammatory diseases such as OA. To determine the effect of ivabradine on activation of NF-κB, we exposed HPCs to 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h and measured both nuclear levels of p65 protein and luciferase activity of NF-κB. As shown in Fig. 8A, Western blot analysis revealed that ivabradine treatment mitigated nuclear translocation of p65 in a dose-dependent manner. As shown by the results of luciferase reporter assay, this also reduced TNF-α-induced activation of NF-κB in a dose-dependent manner (Fig. 8B).

3.8. The protective effects of Ivabradine against TNF-α-induced degradation of ECM are mediated by HCN2

To further investigate whether the effects of ivabradine are mediated by the HCN channel, we knocked down the expression of HCN2. Human primary chondrocytes were transducted with HCN2 shRNA lentivector. 12 h later, cells were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20 nM) for 24 h. Successful knockdown of HCN2 was shown in Fig. 9A. Importantly, knockdown of HCN2

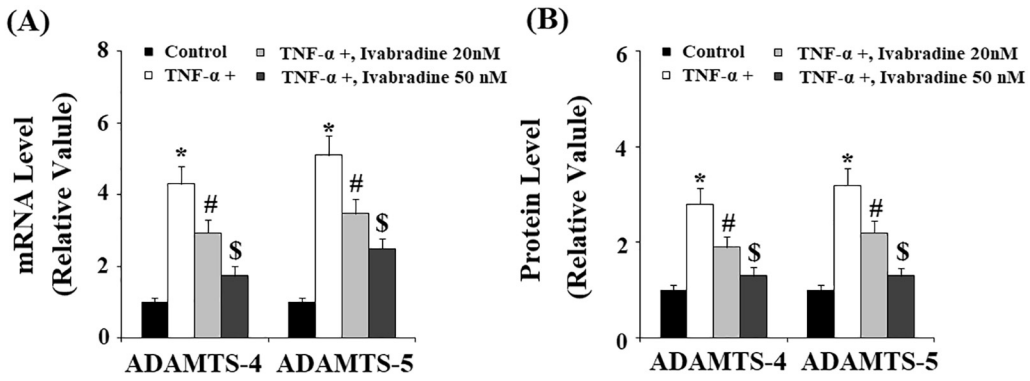

Fig. 4. Ivabradine abrogated the expression of ADAMTS-4 and ADAMTS-5 induced by TNF-α in human primary chondrocytes. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. (A). mRNA levels of ADAMTS-4 and ADAMTS-5 were determined by real-time PCR; (B). Protein levels of ADAMTS-4 and ADAMTS-5 were determined by ELISA (*, #, $, $P < 0.01$ vs. previous column group).

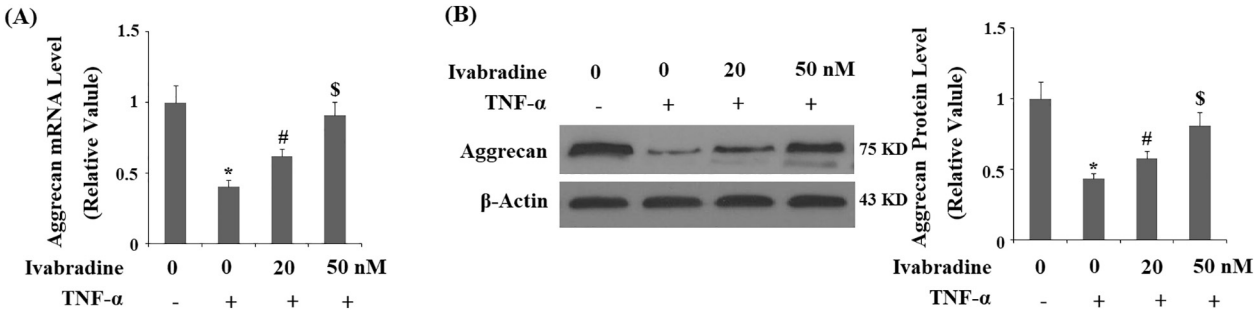

Fig. 5. Ivabradine mitigated TNF-α-induced degradation of aggrecan in human primary chondrocytes. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. Expression of aggrecan was determined by Western blot analysis (*, #, $, P < 0.01 vs. previous column group).

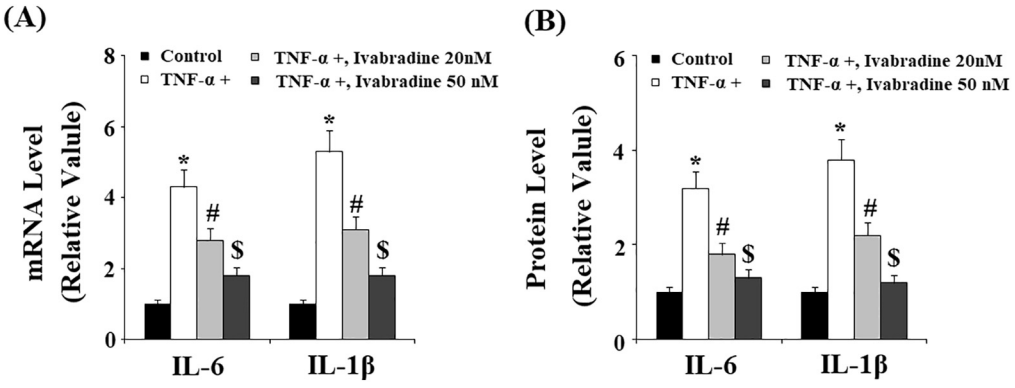

Fig. 6. Ivabradine mitigated TNF-α-induced production of IL-6 and IL-1β. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. (A). Expressions of IL-6 and IL-1β at the mRNA level were determined by real-time PCR; (B). Expressions of IL-6 and IL-1β at the protein level were determined by ELISA (*, #, $, P < 0.01 vs. previous column group).

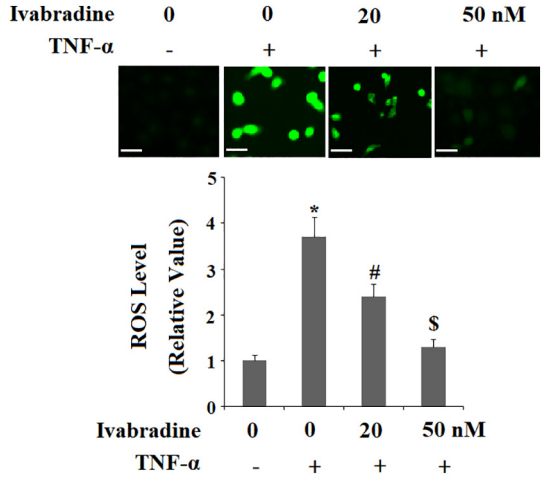

Fig. 7. Ivabradine mitigated TNF-α-induced generation of ROS. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. Intracellular ROS was determined by DCFH-DA assay. Scale bar, 100 μm (*, #, $, P < 0.01 vs. previous column group).

enhanced the protective effects of ivabradine against TNF-α- induced degradation of both type II collagen and aggrecan (Fig. 9B). These findings suggested that the inhibitory effects of ivabradine in ECM degradation might be mediated by HCN2.

4. Discussion

OA is regarded as a major global health problem that primarily affects elderly patients and takes a considerable toll on quality of life and mobility. In 2018, the Centers for Disease Control (CDC) reported that over 30 million people in the US alone suffer from OA, while this number is forecasted to increase to over 70 million by 2030 [12]. Presently, arthroplasty is the preferred therapy for late stage OA and has a success rate of over 85% [13]. However, this treatment is highly invasive and causes other burdens for patients, especially when old age is a factor, which can prolong healing time and increase susceptibility to infection and other complications. While there is a clear need for novel drug therapies against the development and progression of OA, the molecular mechanisms behind the disease are complicated. In the present study, we stimulated human primary chondrocytes with the pro-inflammatory cytokine TNF-α to mimic OA conditions and then set out to determine the effects of ivabradine on the increased expression of MMPs, ADAMTS, IL-6, IL-1β, generation of ROS, and activation of NF-κB induced by TNF-α.

Research has found that cartilage destruction in OA involves a wide range of complex factors including MMPs and other cytokines such as IL-6, IL-1β and TNF-α [14]. Also termed “collagenases”, MMPs are a class of degradative enzymes that target type II collagen, the main structural component of the articular cellular matrix. Of these, MMP-3 and MMP-13 are recognized as playing key roles in OA. Similarly, ADAMTS are a family of aggrecanases that cleave proteoglycans and aggrecans, with ADAMTS-4 cleaving the Glu373-Ala374 bond in aggrecan [15; 16]. Under normal physiological conditions, MMPs and ADAMTS are released at relatively low levels and maintain homeostasis through regular matrix turnover. However, it has been well-demonstrated that release of both MMPs and ADAMTS is increased in OA chondrocytes, thereby contributing to excessive degradation of the ECM [17]. Here, we found that treatment with 10 mg/ml TNF-α significantly increased expression of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 in HPCs. This effect was abrogated by exposure to 20 and 50 ng ivabradine in a dose-dependent manner (Figs. 2 and 4). Next, we confirmed this result by observing the effect of ivabradine on destruction of type II collagen and aggrecan resulting from TNF-α-induced upregulation of MMPs and ADAMTS, respectively. As shown in Figs. 3 and 5, exposure to ivabradine at the concentrations of 20 and 50 ng indeed ameliorated degradation of type II collagen and aggrecan induced by TNF-α. These

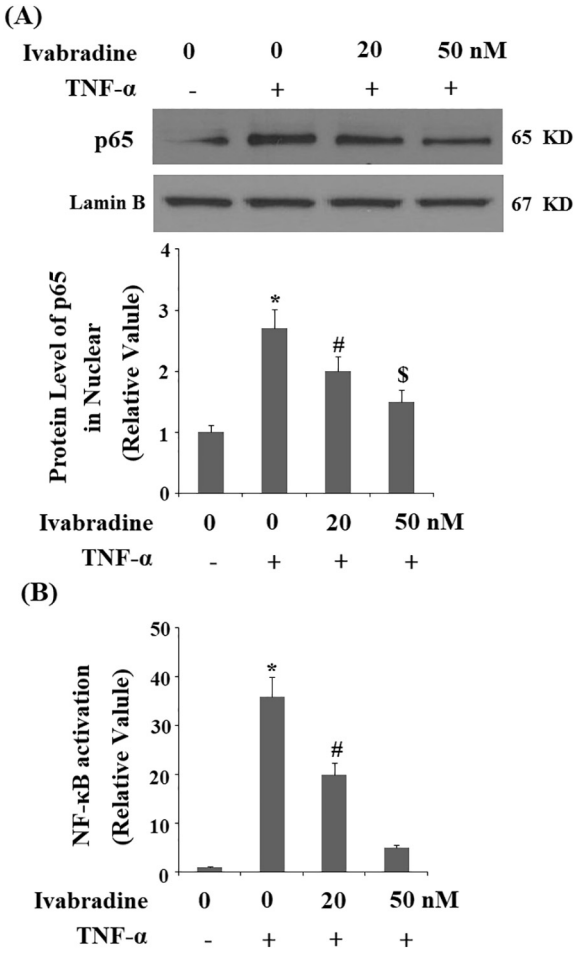

Fig. 8. Ivabradine mitigated TNF-α-induced activation of NF-κB. Human primary chondrocytes were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20, 50 nM) for 24 h. (A). Nuclear level of p65; (B). Luciferase reporter assay demonstrated that ivabradine treatment suppressed NF-κB activation in a dose-dependent manner (*, #, $, P < 0.01 vs. previous column group).

findings imply that treatment with ivabradine may be able to mediate destruction of the articular ECM by downregulating expression of MMPs and ADAMTS.

Interleukins are a class of proinflammatory cytokines that have been shown to play critical roles in a variety of chronic inflammatory diseases including OA. Of these, IL-6 and IL-1β have been shown to be involved in the pathogenesis of OA [18;19]. IL-1β is released in response to oxidative stress stimuli and the resulting generation of ROS, which in turn upregulates the release of IL-6 [19]. This response is mediated via activation of the NF-κB signaling pathway [20;21]. It was recently demonstrated that blockade of IL-1β via the NF-κB pathway may attenuate OA, making this pathway a target of particular interest [22]. In the present study, we found that exposure to TNF-α significantly increased expression of IL-1β and IL-6 in HPCs as well as generation of ROS. Remarkably, upregulation of these cytokines by TNF-α was abrogated by treatment with 20 and 50 ng ivabradine in a dose-dependent manner at both the mRNA and protein levels (Fig. 6). Furthermore, treatment with ivabradine also significantly reduced the increased production of ROS by HPCs stimulated with TNF-α as demonstrated by DCFH-DA staining (Fig. 7). To determine whether this inhibitory effect on expression of IL-1β and IL-6 is exerted via NF-κB signaling, we measured nuclear translocation and accumulation of p65 protein as well as activation of NF-κB. As shown in Fig. 8, stimulation with TNF-α indeed increased nuclear accumulation of p65, which was ameliorated by 20 and 50 ng ivabradine in a dose-dependent manner. Concordantly, TNF-α-induced luciferase activity of NF-κB was also significantly decreased upon treatment with 20 and 50 ng ivabradine in a dose-dependent manner.

Under normal conditions, articular chondrocytes maintain a dynamic equilibrium between synthesis and degradation of ECM components, including type II collagen and aggrecan, the most abundant proteoglycan in articular cartilage [23]. In osteoarthritic states, there is usually an increase in both degradation and synthesis of ECM molecules within the joint, with an overall shift toward catabolism over anabolism [24]. In addition to inducing degradation of the ECM, TNF-α plays an important role in inhibiting ECM synthesis. After activates its receptor on cell membrane, TNF-α could inhibit ECM synthesis by suppressing expression of matricellular protein connective tissue growth factor (CCN2/CTGF), which is an important regulator of cellular adhesion, proliferation, migration, and ECM synthesis [25]. An important limitation of the current study is that the molecular mechanisms whereby ivabradine inhibits TNF-α- induced reduction of mRNA levels of type II collagen and aggrecan are still unknown. Another limitation is that all of our findings are based on in vitro experiments. Indeed, OA is a multifaceted musculoskeletal disease with complicated pathological characteristics. The long experimental time is needed for OA development. Therefore, current preclinical research is largely dependent on animal models. Therefore, future study with ideal animal models of OA will provide a more complete picture.

The results of the present study demonstrate that ivabradine, a well-tolerated and relatively new selective I_f inhibitor, may have potential in the treatment of OA by virtue of its ability to abrogate TNF-α-induced expression of MMPs and ADAMTS, degradation of type II collagen and aggrecan, upregulation of IL-1β and IL-6, generation of ROS, accumulation of p65 and subsequent activation of the NF-κB pathway. Further

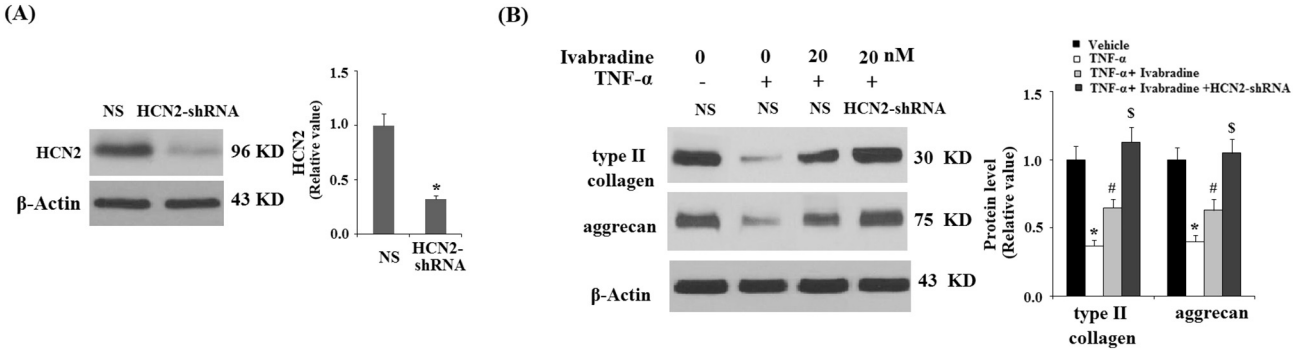

Fig. 9. The inhibitory effects of ivabradine in extracellular matrix degradation might be mediated by the HCN channel. Human primary chondrocytes were transducted with HCN2 shRNA lentivector. 12 h later, cells were treated with 10 ng/ml TNF-α in the presence or absence of ivabradine (20 nM) for 24 h. (A). Western blot analysis of HCN2; NS, non-specific shRNA; (B). Western blot analysis of type II collagen and aggrecan (*, #, $, P < 0.01 vs. previous column group).

research is needed to elucidate the exact mechanisms through which ivabradine exerts the beneficial effects demonstrated in this study.

References

[3] G. Zuo, X. Ren, Qian X2, P. Ye, J. Luo, X. Gao, J. Zhang, S. Chen, Inhibition of JNK and p38 MAPK-mediated inflammation and apoptosis by ivabradine improves cardiac function in streptozotocin-induced diabetic cardiomyopathy, J. Cell. Physiol. (2018), https://doi.org/10.1002/jcp.27070.

[4] B. Li, J. Zhang, Z. Wang, S. Chen, Ivabradine prevents low shear stress induced endothelial inflammation and oxidative stress via mTOR/eNOS pathway, PLoS One 11 (2) (2016) e0149694.

[5] H.M. Ismail, J. Miotla-Zarebska, L. Troeberg, X. Tang, B. Stott, K. Yamamoto, H. Nagase, A.J. Fosang, T.L. Vincent, J. Saklatvala, Brief report: JNK-2 controls aggrecan degradation in murine articular cartilage and the development of experimental osteoarthritis, Arthritis Rheum. 68 (2016) 1165–1171.

[6] Chuan-Yueh Huang, Kuan-Yui Lai, Li-Feng Hung, Wu Wan-Lin, Feng-Cheng Liu, Ling-Jun Ho, Advanced glycation end products cause collagen II reduction by activating Janus kinase/signal transducer and activator of transcription 3 pathway in porcine chondrocytes, Rheumatology 50 (8) (1 August 2011) 1379–1389.

[7] I. Tchetverikov, L.S. Lohmander, N. Verzijl, T.W.J. Huizinga, J.M. Tekoppele, R. Hanemaaijer, J. Degroot, MMP protein and activity levels in synovial fluid from patients with joint injury, inflammatory arthritis, and osteoarthritis, Ann. Rheum. Dis. 64 (5) (2005) 694–698.

[8] P. Verma, K. Dalal, ADAMTS-4 and ADAMTS-5: key enzymes in osteoarthritis, J. Cell. Biochem. 112 (12) (2011 Dec) 3507–3514, https://doi.org/10.1002/jcb.23298.

[9] P. Lepetsos, A.G. Papavassiliou, ROS/oxidative stress signaling in osteoarthritis, Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1862 (4) (2016) 576–591.

[10] S.N. Wang, G.P. Xie, C.H. Qin, Y.R. Chen, K.R. Zhang, X. Li, ... B. Yu, Aucubin prevents interleukin-1β induced inflammation and cartilage matrix degradation via inhibition of NF-κB signaling pathway in rat articular chondrocytes, Int. Immunopharmacol. 24 (2) (2015) 408–415.

[11] Chen LY1, Lotz M2, R. Terkeltaub, R. Liu-Bryan, Modulation of matrix metabolism by ATP-citrate lyase in articular chondrocytes, J. Biol. Chem. 293 (31) (2018) 12259–12270.

[12] Osteoarthritis [Online], Centers for Disease Control and Prevention Centers for Disease Control and Prevention, (2018).

[13] N. Rosshirt, T. Engbarth, T. Gotterbarm, S. Hagmann, B. Moradi, M1/M2 macrophages induce chondral MMP/ADAMTS enzyme secretion in a direct co-culture experiment, Osteoarthr. Cartil. 26 (Supplement 1) (April 2018) S129.

[14] G.Y. Li, R.F. Zhang, L. Pan, X. Zhang, et al., A study of serum cartilage oligomeric matrix protein and matrix metalloproteinase-3 concentration in osteoarthritic rabbit models, Zhonghua Nei Ke Za Zhi 50 (2011) 416–419.

[17] X.Q. Chu, J.J. Wang, L.D. Dou, G. Zhao, Cartilage oligomeric matrix protein and matrix metalloproteinase-3 expression in the serum and joint fluid of a reversible osteoarthritis rabbit model, Genet. Mol. Res. 14 (4) (2015) 14207–14215.

[19] M.S. Makki, T.M. Haqqi, Histone deacetylase inhibitor vorinostat (SAHA) suppresses IL-1β–induced matrix metallopeptidase-13 expression by inhibiting IL-6 in osteoarthritis chondrocyte, Am. J. Pathol. 186 (10) (2016) 2701–2708.

[22] X. Liu, H. Fang, C. Zeng, D. Cai, Myricetin attenuates osteoarthritis by blockade of the IL-1β/MAPK pathway, Osteoarthr. Cartil. 26 (2018) S84.

[23] F. Guilak, R.J. Nims, A. Dicks, C.L. Wu, I. Meulenbelt, Osteoarthritis as a disease of the cartilage pericellular matrix, Matrix Biol. 71-72 (2018 Oct) 40–50.

[24] A.S. Lee, M.B. Ellman, D. Yan, J.S. Kroin, B.J. Cole, A.J. van Wijnen, H.J. Im, A current review of molecular mechanisms regarding osteoarthritis and pain, Gene 527 (2) (2013) 440–447.

[25] Z.I. Johnson, Z.R. Schoepflin, H. Choi, I.M. Shapiro, M.V. Risbud, Disc in flames: roles of TNF-α and IL-1β in intervertebral disc degeneration, Eur. Cell. Mater. 30 (2015) 104–116.