

ORIGINAL ARTICLE

Chronic Calcium Channel Inhibitor Verapamil Antagonizes TNF- α -Mediated Inflammatory Reaction and Protects Against Inflammatory Arthritis in Mice

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Abstract—It is well established that the tumor necrosis factor- α (TNF- α) plays a dominant role in rheumatoid arthritis (RA). Calcium channel is recently reported to be closely associated with various inflammatory diseases. However, whether chronic calcium channel blocker verapamil plays a role in RA still remains unknown. To investigate the role of verapamil in antagonizing TNF- α -mediated inflammation reaction and the underlying mechanisms, bone marrow-derived macrophages (BMDM) cells were cultured with stimulation of TNF- α , in the presence or absence of verapamil. Inflammation-associated cytokines, including IL-1, IL-6, inducible nitric oxide synthase 2 (NOS-2), and cyclooxygenase-2 (COX-2), were assessed, and verapamil suppressed TNF- α -induced expression of inflammatory cytokines. Furthermore, collagen-induced arthritis (CIA) mice models were established, and arthritis progression was evaluated by clinical and histological signs of arthritis. Treatment of verapamil attenuated inflammation as well as joint destruction in arthritis models. In addition, activity of NF- κ B signaling pathway was determined both *in vitro* and in mice arthritis models, and verapamil inhibited TNF- α -induced activation of NF- κ B signaling both *in vitro* and in mice models. Collectively, chronic calcium channel blocker verapamil may shed light on treatment of inflammatory arthritis and provide a potential therapeutic instrument for RA in the future.

KEY WORDS: verapamil; TNF- α ; NF- κ B signaling; collagen-induced arthritis.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by pain, joint inflammation, progressive cartilage damage, and bone erosion. Inflammatory cytokines produced by innate immune cells, in particular tumor necrosis factor α (TNF- α), play a critical role in the pathogenesis of inflammatory arthritis [1, 2]. Indeed, several approved biological agents, including anti-TNF antibodies (infliximab, adalimumab) and a recombinant soluble TNF receptor-Fc fusion protein (etanercept) showed promising therapeutic effects in patients with RA and other inflammatory diseases [3]. However, considering the cost of such biologic agents, potential resistance of these agents, and their limited efficacy, there is still a great need for the investigation of novel therapeutic mechanisms and agents [4, 5].

Calcium channel is expressed extensively within organism and plays a vital role in various diseases [6, 7]. Recently, it was reported that an inhibitor of store-operated calcium channel (SOC), an ion channel which displays selective permeability to calcium ions, attenuated rheumatoid arthritis in mice models [8]. However, it is unknown whether inhibitor of another calcium channel, chronic calcium channel, also protects against RA development. Verapamil is a commonly accepted chronic calcium channel inhibitor in cardiac diseases [9]. Recently, verapamil was reported to antagonize production as well as function of TNF- α and protect against inflammation reaction [10–12]. It is well-established that nuclear factor-kappa B (NF-kB) signaling plays a detrimental role in progress of inflammation [13] and is closely associated with RA development. It is known that verapamil inhibits activation of NF-kB signaling [14]. Recently, it was reported that verapamil protects against cartilage destruction in mice osteoarthritis models [15]. However, whether verapamil acts as a potential treatment of RA remains to be elucidated. The purpose of this study is to determine (1) whether verapamil is able to block the inflammatory function of TNF- α and has therapeutic effect in inflammatory arthritis and, if so, (2) what underlying mechanisms are involved.

MATERIAL AND METHODS

Mice

All animal studies were performed in accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee of Shandong University. DBA/1J mice were obtained from Jackson Laboratories.

Isolation and Culture of Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages (BMDM) cells were isolated from wild-type mice as previously reported [16]. Cells were isolated of bone marrow cells from mouse femurs and tibias. And, BMDM cells were cultured with DMEM containing 10 % fetal bovine serum, 1 % penicillin and streptomycin, and 15 % L929 conditioned media. Cells were differentiated to macrophages after 7 days of incubation at 37 °C.

CIA model

The collagen-induced arthritis (CIA) mice model was established as we have previously reported [17, 18]. Briefly, 10-week-old male DBA1/J mice were immunized via a 0.1-ml intradermal injection at the base of the tail with 100- μ g chicken type II collagen (Chondrex, LLC, Seattle, WA) emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 4 mg/ml heat denatured mycobacterium (Chondrex, LLC, Seattle, WA) (day 0). A second injection was given subsequently after 21 days as previously reported [18]. To determine protective effects, 20 mg/kg verapamil [19] was administered intraperitoneally every day starting on day 21 [20, 21].

Evaluation for Clinical Arthritis

Clinical signs of arthritis in CIA mice models were evaluated to determine arthritis incidence. Each paw was evaluated and scored individually using a 0 to 4 scoring system. The paw scores were summed to yield individual mouse scores, with a maximum score of 16 for each animal as published previously [17].

Histopathological Examination of Joints

Following routine fixation, decalcification, and paraffin embedding, tissue sections were prepared and stained with hematoxylin and eosin. Decalcification procedure was performed as previously reported [18]. Briefly, paw samples from mice were put into 10 % EDTA, and the decalcification procedure lasts for demineralization. All slides were coded and submitted for evaluation by investigators blinded to the experimental conditions. The extent of synovitis, pannus formation, and bone/cartilage destruction was determined using a graded scale as follows: grade 0, no signs of inflammation; grade 1, mild inflammation with hyperplasia of the synovial lining without cartilage destruction; and grades 2 through 4, increasing degrees of inflammatory cell infiltrate and bone destruction. Sections were also stained with 0.1 % Safranin O to study the joint pathologic features. PG depletion was scored on an arbitrary scale of 0–3, where 0 = normal, 1 = fully stained cartilage, 2 = detained cartilage, and 3 = completely depleted of PGs. The degree of chondrocyte death was scored on a scale of 0–3, where 0 = no empty lacunae and 3 = complete loss of chondrocytes in the cartilage layer. Cartilage surface erosion was scored on a scale of 0–3, where 0 = no cartilage loss and 3 = complete loss of articular cartilage. All the analyses of cartilage destruction were done as previously reported [22, 23]. Histopathologic changes were scored on five semi-serial sections,

spaced 140 mm apart. Scoring was performed in a blinded manner by two independent observers. In addition, TRAP staining was performed for detection of osteoclast activity as previously reported [13].

Western Blot

Bone marrow-derived macrophages (BMDM) cells were isolated from wild-type mice and stimulated with 10 ng/ml TNF- α , in the presence or absence of indicated doses of verapamil. At different time points, total cell lysate or cytoplasmic and nuclear extracts were prepared following our published protocol [19]. Cell extracts (~30 μ g) were resolved on a 10 % SDS-PAGE gel and probed using specific antibodies against total as well as phosphorylated I κ B α , NF- κ B p65, and NOS-2. Synovium from knee joints of CIA models was harvested and homogenized, followed by Western blot for NOS-2. β -Tubulin was used as internal control in all this set of experiments.

Reporter Gene Assay

To examine whether verapamil inhibits TNF-mediated transactivation of NF- κ B-dependent reporter genes, reporter gene assay was performed in a method as described before with modifications [24]. Briefly, BMDM cells grown to ~50 % confluence were transfected with 1 μ g of the p6XNF- κ B-Luc reporter plasmid and 1 μ g of the pSVGal plasmid (internal control), using Lipofectamine 2000 Reagent (Invitrogen). Forty-eight hours following transfection, the cells were starved overnight and then stimulated for 6 h with 10 ng/ml TNF- α in the presence or absence of various doses of verapamil. Luciferase activity was then measured.

Immunofluorescence Staining of NF- κ B p65

BMDM cells were cultured on coverslips, stimulated with 10 ng/ml TNF- α in the presence or absence of verapamil for 1 h. Then, immunofluorescence staining of NF- κ B p65 was performed on these cells as described previously and examined using a confocal fluorescence microscope system [22].

Immunohistochemistry

The metatarsal joints of CIA mice treated with PBS or verapamil were sectioned, deparaffinized, rehydrated, and incubated in Tris buffer (10 Mm Tris-HCl (pH 8.0), 150 mM NaCl). The samples were then incubated with rabbit anti-mouse phosphorylated I κ B α (1:100) at 4 °C overnight. Then, the sections were incubated for 30 min with biotinylated anti-rabbit IgG (Vector, Burlingame, CA) and subsequently stained using a biotin-streptavidin-peroxidase

protocol (Vector). Horseradish peroxidase (HRP) activity was detected using 3,3'-diaminobenzidine and H₂O₂. Slides were counterstained with 0.5 % methyl green.

Real-Time PCR

Total RNA was extracted from synovium of knee joint in PBS or verapamil-treated CIA models, and BMDM cells stimulated with TNF- α (10 ng/ml) in the presence or absence of verapamil (5 μ M) for 4 h using RNeasy kit (Qiagen), and first-strand complementary DNA (cDNA) was generated with ImProm-II reverse transcription system (Promega). Real-time PCR was performed with SYBR Green I dye used to monitor DNA synthesis. Data from each sample were normalized to GAPDH. Primers used for real-time RT-PCR were designed to generate products between 100 and 200 bp in length. Oligonucleotides used as the specific primers to amplify mouse genes are as follows: Total mRNA was isolated from. Then, they were reverse-transcribed to cDNA. Real-time PCR was performed with the following sequence-specific primers: 5'-TACAAGCTGGCTGGTGGGA-3' and 5'-GTCGCGGTCTCAGGACCTT-3' for NF- κ B2; 5'-AATCTCACAGCAGCACATCA-3' and 5'-AAGG TGCTCATGTCTCATC-3' for interleukin-1 β (IL-1 β); 5'-CCTTCTACCCCAATTTCCAAT-3' and 5'-GCCA CTCCTTCTGTGACTCCAG-3' for IL-6; 5'-ACA GGAGGGGTTAAAGCTGC-3' and 5'-TTGT CTCCAAGGGACCAGG-3' for NOS-2; 5'-AAT GCT GAC TAT GGC TAC AAA A-3' and 5'-AAA ACT GAT GCG TGA AGT GCT G-3' for COX-2; 5'-CTTC ACCACCATGGAGAAGGC-3' and 5' GACGGACA CATTGGGGGTAG-3' for GAPDH. The presence of a single-specific PCR product was verified by melting curve analysis, and for each gene, the experiments were repeated three times.

Micro-CT

The microstructure destruction of metatarsal bones was measured using a Scanco μ CT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) as we have previously reported [13, 25]. A threshold of 300 was used for evaluation of all scans. The whole hind paw was scanned, and 3-D reconstruction was performed to evaluate bone erosion of metatarsal joints.

Assays for Circulating IL-1 β and IL-6

Serum was collected from CIA model, and levels of IL-1 β as well as IL-6 were assayed through ELISA as previously reported [24]. IL-1 β and IL-6 were assayed

using a commercial kit (eBioscience), according to the instructions of the manufacturer.

Statistical Analysis

For comparison of treatment groups, we performed unpaired *t* tests (Mann–Whitney), paired *t* tests, and one-way or two-way ANOVA (where appropriate). For ANOVA, we used Bonferroni *post hoc* analysis to compare treatment groups. All statistical analysis was performed using GraphPad Prism Software (version 4.01). Statistical significance was achieved when $P < 0.05$.

RESULTS

Verapamil Attenuated Inflammatory Reaction Induced by TNF- α *In Vitro*

To determine whether verapamil antagonizes TNF- α function *in vitro*, BMDM cells were stimulated with 10 ng/ml TNF- α for 4 h in the presence or absence of 5 or 20 μ M

verapamil, followed by real-time RT-PCR assay. As shown in Fig. 1a–d, TNF- α -mediated inflammatory cytokine production, including IL-1 β , IL-6, NOS-2, and COX-2, was largely abolished by additional use of verapamil in a dose-dependent manner. Furthermore, BMDM cells were cultured with 10 ng/ml TNF- α for 24 h, with or without treatment of 5 or 20 μ M verapamil, followed by Western blot assay. Figure 1e shows that TNF- α significantly elevated expression levels of NOS-2 and COX-2, while this effect of TNF- α was remarkably diminished by additional treatment of verapamil dose dependently. Moreover, levels of interleukin (IL)-1 and IL-6 in conditional medium of the indicated groups were measured through ELISA. As shown in Fig. 1f–g, both mentioned inflammatory mediators were greatly antagonized by administration of 5 and 20 μ M verapamil.

Verapamil Protects Against Development of Arthritis in CIA Mice Models

To define the protective role of verapamil *in vivo*, we induced CIA model in 8–10-week-old male DBA/1J mice.

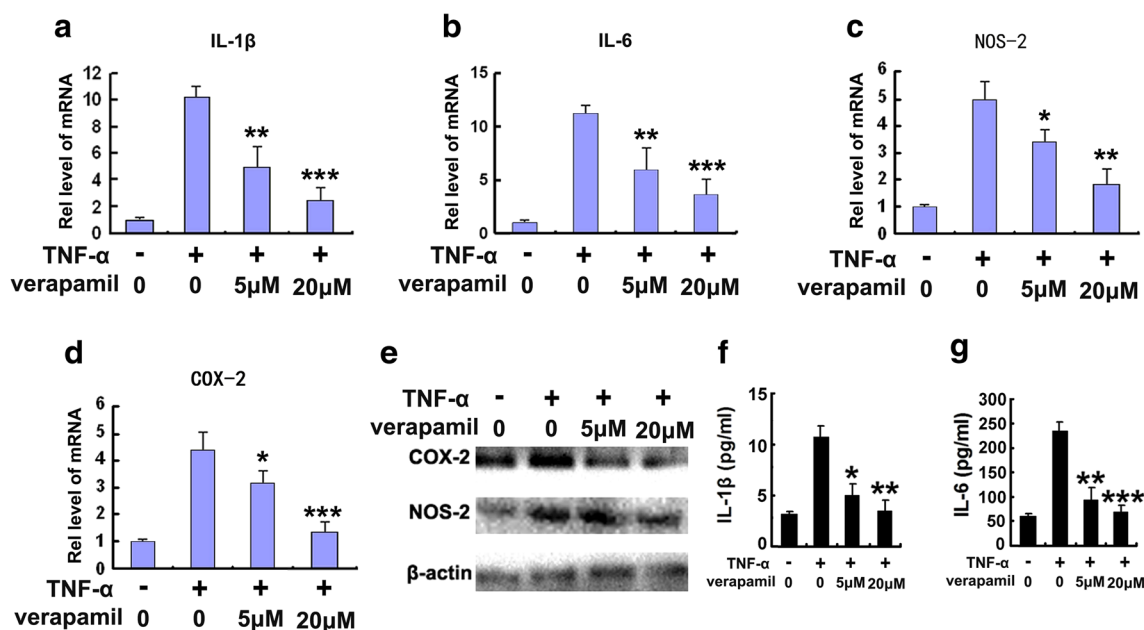


Fig. 1. Verapamil antagonizes TNF- α -induced inflammatory reaction in BMDM cells. **a–d** Verapamil inhibited TNF- α -mediated production of IL-1 β , IL-6, NOS-2, and COX-2 in BMDM cells, assayed by real-time RT-PCR. BMDM cells were incubated with TNF- α (10 ng/ml) in the presence or absence of verapamil (5 or 20 μ M) for 4 h, cell extract was then harvested and assessed by real-time PCR. **e** Expression of NOS-2 as well as COX-2 in PBS and verapamil (5 or 20 μ M) treatment group following TNF- α stimulation, assayed by Western blot. Protein extracts were collected from BMDM cells 24 h following TNF- α (10 ng/ml) stimulation in indicated treatment groups, and Western blot was performed. **f, g** Expression levels of IL-1 β and IL-6 in TNF- α (10 ng/ml)-stimulated BMDM cells were significantly diminished by verapamil (5 or 20 μ M), as measured through ELISA. Conditional media were collected from all the indicated groups of BMDM cells, and ELISA was performed for IL-1 β and IL-6. The values are the mean \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ vs. PBS treatment group.

Twenty-one days after immunization, the mice were divided into two groups based on their treatments: the CIA control group (PBS, $n = 7$) and the experimental groups (verapamil, 20 mg/kg, $n = 7$). Intraperitoneal injection of each agent was performed every day, and macrograph of hind paw (Fig. 2a) as well as clinical score of arthritis (Fig. 2b) implied inflammation in CIA model, while administration of verapamil remarkably attenuated development of arthritis. Forty-two days following immunization, samples of metatarsal joints from each group were collected and histological analyses as well as micro CT assay were performed. As shown in Fig. 2c, d, HE staining of metatarsal joints indicated severe synovitis, pannus, and erosion sites in CIA models. By contrast, verapamil dramatically alleviated inflammation in the arthritis model. Furthermore, synovium samples of metatarsal joints were collected from each group, and messenger RNA (mRNA) and protein were isolated. As revealed in Fig. 2e–h, mRNA levels of inflammation-associated molecules in CIA model, including IL-1 β , IL-6, NOS-2, and COX-2, were significantly reduced by verapamil treatment. Figure 2i, j indicates that verapamil markedly reduced expression of NOS-2 and COX-2 compared with PBS group in CIA model. Moreover, serum was collected from each group, and IL-1 β and IL-6 levels were assayed through ELISA. Figure 2k, l shows that circulating levels of both inflammatory molecules were greatly diminished in CIA with treatment of verapamil. Collectively, this set of experiments suggests that verapamil plays a protective role in CIA.

Cartilage destruction and inflammatory bone resorption are well accepted characteristics of rheumatoid arthritis [26, 27]. In this study, metatarsal joints were collected from each group, and Safranin O staining was performed. As indicated in Fig. 3a, serious destruction of cartilage was observed in CIA model, while the destruction was dramatically protected through verapamil. Based on Safranin O staining, severity of cartilage destruction was further testified through a histological scoring system. Figure 3b–d reveals that cartilage PG depletion, chondrocyte death, and cartilage surface erosion in CIA model were greatly attenuated in verapamil treatment group. ADAMTS-5 and MMP-13 are well accepted molecules for cartilage destruction in arthritis [28, 29]. In the current study, metatarsal joints were collected and real-time PCR was performed. Figure 3e shows that levels of ADAMTS-5 and MMP-13 were both diminished in verapamil treatment group. It is reported that calcium channel inhibitors, including verapamil, are associated with osteoclastogenesis [30, 31]. To determine the protective role of verapamil for inflammatory osteoporosis in CIA models, metatarsal joints from each

group were isolated, and micro-CT and TRAP staining were performed. Figure 3f, g indicates that verapamil remarkably protected against inflammatory bone erosion as well as osteoclastogenesis in CIA models. Furthermore, mRNA was collected from bony tissue of metatarsal joints, and Fig. 3h, i reveals that the levels of osteoclast-associated molecules, including TRAP and Cathepsin K were significantly reduced by administration of verapamil.

Verapamil Blocks NF- κ B Signaling Pathway Both *In Vitro* and in the CIA Model

NF- κ B pathway is a critical signaling pathway in various diseases and plays a pivotal role in development and progression of inflammatory arthritis [32]. To investigate whether verapamil inhibits TNF- α -induced stimulation of NF- κ B pathway, BMDM cells were cultured for 3 h in the presence of TNF- α (10 ng/ml), with or without pretreatment of 5 μ M verapamil. Thereafter, cell extract was collected, and real-time PCR was performed. As shown in Fig. 4a, additional use of verapamil suppressed TNF- α -mediated expression of NF- κ B2, a critical composite of NF- κ B signaling pathway. Besides, plasma and nuclear proteins were collected respectively as described before for testing nuclei translocation of NF- κ B p65. Figure 4b demonstrates that verapamil inhibited TNF- α -induced nuclei translocation of NF- κ B p65. To further assess the role of verapamil in inhibiting activation of NF- κ B signaling, immunostaining for NF- κ B p65 was performed on BMDM cells cultured in the presence of 10 ng/ml TNF- α for 1 h, in the absence or presence of verapamil. As indicated in Fig. 4c, verapamil inhibited TNF- α -mediated activation of NF- κ B-dependent reporter gene by measuring the luciferase activity of increasing concentrations of verapamil (1, 5, 20 μ M) used in BMDM cells. Figure 4d reveals that verapamil suppressed nuclei translocation of NF- κ B p65. Additionally, activation of NF- κ B signaling in the synovium fibroblast of tarsal joint in CIA model was assessed through immunohistochemistry of pI κ B- α . As shown in Fig. 4e, verapamil suppressed expression level of NF- κ B2 in prevention experiment of CIA model was measured by real-time PCR. Figure 4f indicates the protective role of verapamil in the activation of NF- κ B signaling in CIA model by histopathological examination.

DISCUSSION

Verapamil has been widely used and is well-established in clinic for the safety and efficacy in various diseases [33]. Recently, it was reported that verapamil was

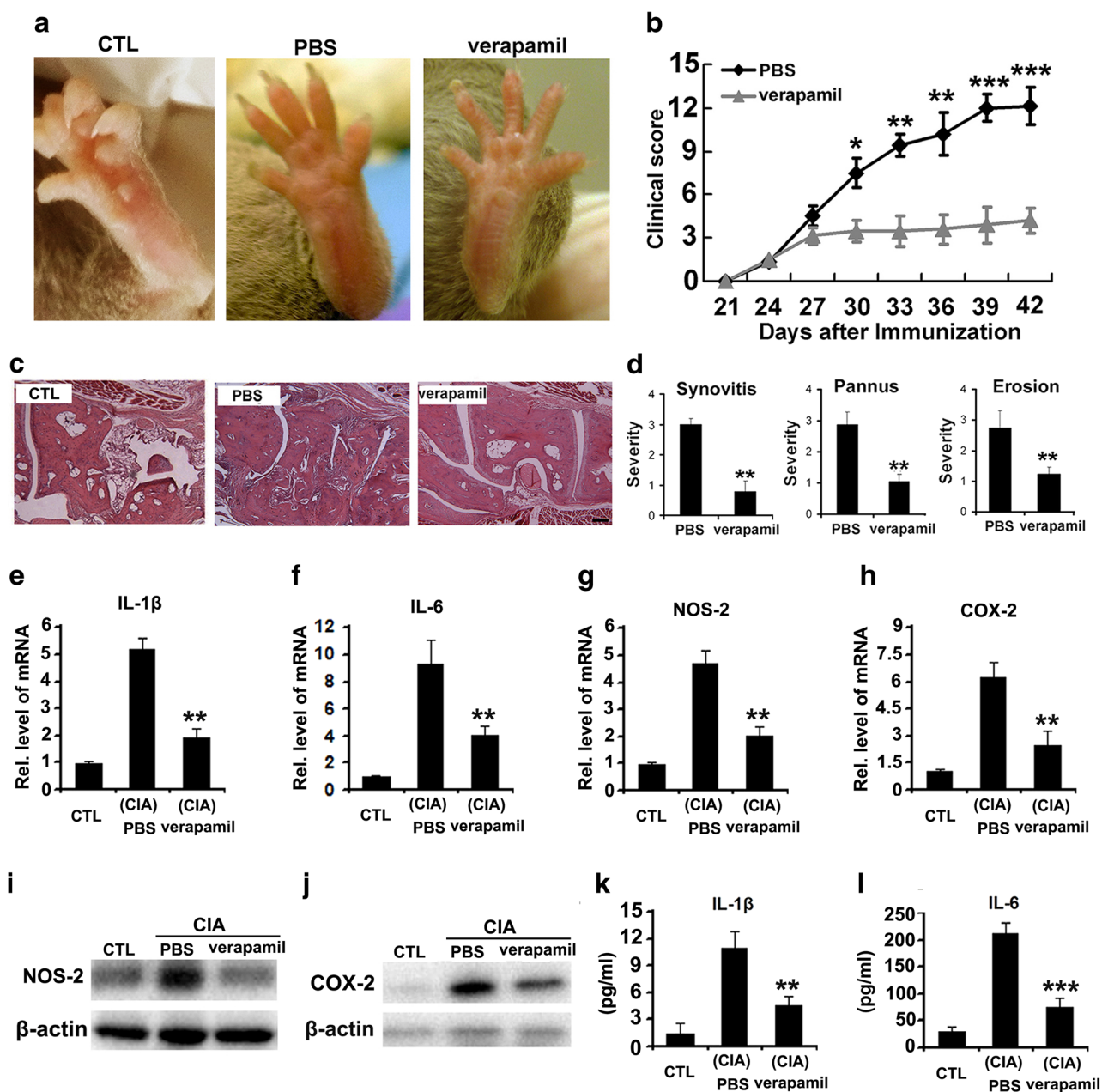


Fig. 2. Verapamil protects against CIA development. **a** Representative photographs of hind paws from normal control and CIA mice treated with PBS or verapamil. DBA/1J mice were induced for CIA model. Twenty-one days after immunization, PBS (negative control, $n=7$) or verapamil ($n=7$) were administered every day in a dose of 20 mg/kg body weight through intraperitoneal injection until day 42. **b** Clinical arthritis scores in PBS or verapamil-treated CIA mice. The experiment process is the same as **a**. Data are presented as the mean clinical score \pm s.e.m. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus the PBS group. **c** Verapamil attenuates severity of inflammation in metatarsal joints of CIA models, as indicated by HE staining. Scale bar = 100 μ m. **d** Evaluation of synovitis, pannus formation, and erosion of tarsal joints in CIA mice. **e-h** IL-1 β , IL-6, NOS-2, and COX-2 gene levels in synovium of CIA model treated with PBS or verapamil, assayed by real-time RT-PCR. RNA extracts were collected from PBS and verapamil-treated CIA model, and real-time RT-PCR was performed. **i, j** Protein levels of NOS-2 and COX-2 in synovium samples of indicated groups, as measured by Western blot. **k, l** Serum levels of IL-1 β and IL-6, as measured by ELISA. Forty-two days after primary immunization and different treatments (started at day 21), serum was taken from normal untreated control mice as well as CIA model of each treatment group, and ELISA was performed for IL-1 β and IL-6. Values are mean \pm s.d. of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus the control PBS group.

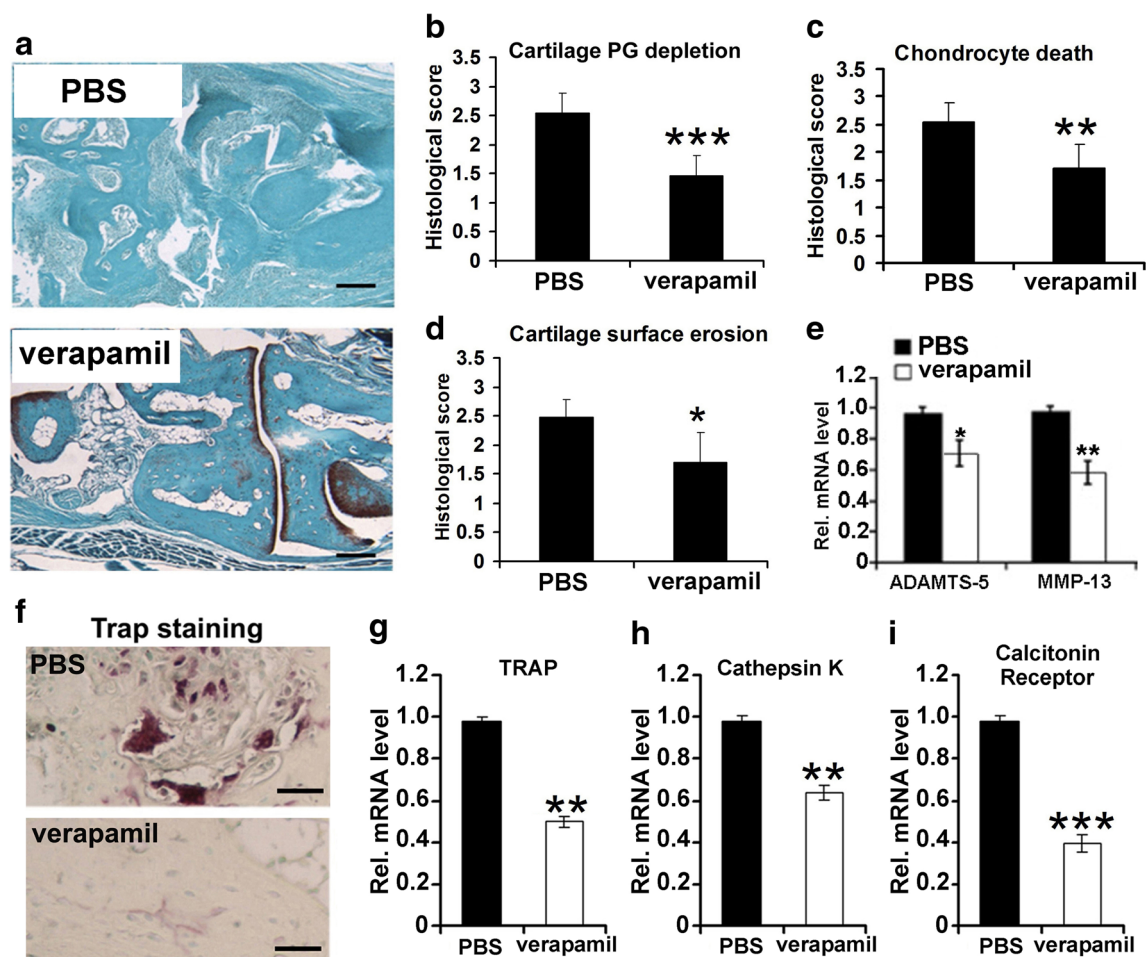


Fig. 3. Verapamil treatment protects against bone erosion and cartilage destruction in CIA model. **a** Verapamil treatment protected cartilage from destruction, assayed by Safranin O staining. Metatarsal joints from both verapamil and PBS-treated CIA model were dissected at day 42 and Safranin O staining was performed. *Scale bar*, 100 μ m. **b–d** Results of histological analysis to determine irreversible cartilage destruction in mice metatarsal joints. On the basis of Safranin O staining, histological scores were determined for the extent of cartilage proteoglycan (PG) depletion, chondrocyte death, and cartilage surface erosion in metatarsal joints from PBS or verapamil treatment group of CIA model. **e** Verapamil treatment diminished expression levels of MMP-13 and ADAMTS-5 in CIA model, as detected by real-time PCR. Metatarsal joints from CIA mice were collected at day 42 and real-time PCR was performed. **f** Verapamil therapy attenuated bone erosion in CIA model, assessed by micro-CT. Then, hind paws from each group were dissected and micro-CT was performed. **g** Verapamil suppressed inflammatory osteoclastogenesis in CIA model, assayed by TRAP staining. Hind paws from **a** were performed with TRAP staining, and osteoclast activity was compared between verapamil and PBS group. *Scale bar*, 50 μ m. **h, i** Verapamil treatment diminished osteoclastic biomarkers TRAP and Cathepsin K in CIA model, as assayed by real-time PCR. Values are mean \pm s.d. of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 versus the control PBS group.

able to antagonize inflammation in several pathological conditions and present protective effect [34, 35]. TNF- α is a critical cytokine to induce other inflammation-associated molecules, which further leads to development of inflammatory diseases [36, 37]. TNF- α also promotes osteoclastogenesis and leads to bone erosion in various inflammatory diseases such as rheumatoid arthritis or inflammatory bowel disease [38, 39]. As a predominant inflammatory cytokine, TNF- α activates NF- κ B inflammatory signaling pathway,

stimulates production of other inflammatory cytokines such as IL-1 and IL-6, and enhances tissue destruction in joints, contributing to the aggravating lesion of RA [40]. To date, TNF- α inhibition has become a well-accepted therapeutic strategy for RA [41, 42]. TNF- α neutralizing therapies, including anti-TNF- α monoclonal antibody and soluble TNFR, are well-established instruments in clinic for treatment of rheumatoid arthritis [43–45]. In this study, we have shown that administration of verapamil suppressed the

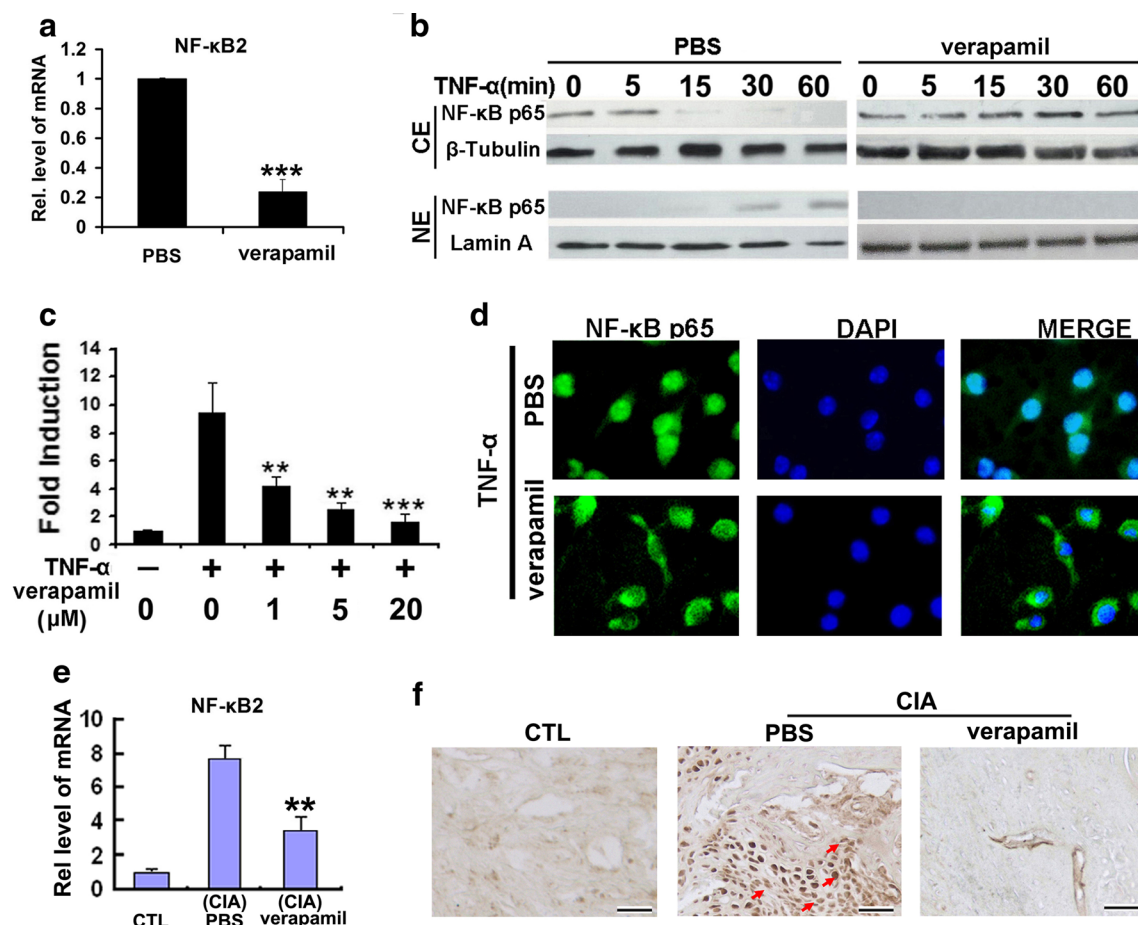


Fig. 4. Verapamil inhibits TNF- α -mediated activation of NF- κ B signaling. **a** Verapamil inhibited TNF- α -induced NF- κ B2 expression. BMDM cells were incubated with TNF- α (10 ng/ml) in the presence or absence of verapamil (5 μ M) for 4 h, and cell extract was then harvested and assessed by real-time PCR. **b** Verapamil impaired TNF- α -mediated activation of NF- κ B signaling, assayed by Western blot. BMDM cells were incubated and treated as Fig. 4a for indicated time points. Then, cytoplasmic (CE) and nuclear (NE) protein extracts were harvested and detected by Western blot. β -Tubulin and lamin A served as cytoplasmic and nuclear controls, respectively. **c** Verapamil inhibited TNF- α -mediated activation of NF- κ B-dependent reporter gene. BMDM cells transfected with the NF- κ B-dependent reporter construct were incubated with TNF- α (10 ng/ml) in the presence of increasing concentrations of verapamil (1, 5, 20 μ M), and the luciferase activity was measured. Values are mean \pm s.d. * P < 0.05; ** P < 0.01; *** P < 0.001 versus TNF- α -stimulated cells. **d** Verapamil suppressed TNF- α -induced nuclear translocation of NF- κ B p65, assayed by immunofluorescence. BMDM cells were stimulated with TNF- α for 4 h, with treatment of PBS or 5 μ M verapamil, then cells were fixed and the location of NF- κ B was visualized by p65 antibody, and the nuclei were stained with DAPI. **e** Verapamil suppressed expression level of NF- κ B2 in prevention experiment of CIA model, as measured by real-time PCR for NF- κ B2. Metatarsal joints of normal control and CIA mice were collected on day 42 after primary immunization with treatment of PBS or verapamil (start on day 21), and then real-time PCR for NF- κ B2 was performed. **f** Verapamil suppressed expression level of pI κ B α in prevention experiment of CIA model, as measured by immunohistochemistry for p-I κ B α . Metatarsal joints of normal control and CIA mice with treatment of PBS or verapamil (start on day 21) were collected on day 42 after primary immunization, and then immunohistochemistry for p-I κ B α was performed. Values are mean \pm s.d. * P < 0.05; ** P < 0.01; *** P < 0.001 versus PBS treatment group. Scale bar, 25 μ m.

inflammation reaction induced by TNF- α *in vitro*. It is known that macrophage plays a pivotal role in the progression of various inflammatory diseases [46, 47]. Macrophage, especially when activated by TNF- α , can strongly exaggerate severity of RA for further production of inflammatory cytokines such as COX-2, NOS-2, IL-1, and IL-6 [48, 49]. In the present study, we took use of BMDM macrophage

and indicated that verapamil suppressed the elevation of the mentioned cytokines mediated by TNF- α , suggesting its protective role in inflammatory reaction.

NF- κ B signaling pathway is a pleiotropic pathway in development of inflammatory diseases and plays an important role in inflammation-associated molecules including NOS-2 as well as COX-2 in RA. Production of these

molecules results in further activation of NF- κ B signaling. Indeed, persistent NF- κ B activation has been observed in both human and animal models of RA. It is reported that verapamil inhibited NF- κ B signaling pathway in the liver [14]. Moreover, the other calcium channel blocker such as nifedipine plays a positive role in vascular inflammation by NF- κ B signaling [50]. In the present study, we found that verapamil affected not only the expression level of NF- κ B signaling but also the production of downstream molecules related with NF- κ B signaling pathway in inflammatory arthritis mice models, which implies the amelioration of verapamil on the RA and other inflammatory diseases.

It is well accepted that cartilage destruction is one feature of RA [51]. Moreover, verapamil is known to protect against osteoarthritis in mice models [15]. In the current study, we established CIA mice models to further verify the chondroprotective role of verapamil *in vivo*. We demonstrated that treatment of verapamil significantly attenuated breakdown of cartilage and reduced the levels of MMP13 as well as ADAMTS-5, which are known to be associated with cartilage degradation in arthritis. Together with the reduced matrix loss of metatarsal joints in verapamil treatment group, all the data suggested the protective effect of verapamil on cartilage matrix in CIA models. Another characteristic of RA is enhanced osteoclastogenesis and bone erosion [52]. Herein, we testified that classical inflammatory osteoclastogenesis and osteolysis in CIA model were greatly attenuated by administration of verapamil. Collectively, these data implied that verapamil may shed light on CIA treatment and serve a potential target for rheumatoid arthritis in the future.

CIA is a most frequently used animal model to study the effect of new therapeutics for RA. Although there are similarities between these conditions, human arthritis conditions still remain to be elucidated. Therefore, CIA only can simulate RA to a certain degree. Furthermore, though verapamil works well in inflammatory arthritis in mice, there is no investigation concerning the improvement of RA symptoms in the patients who used verapamil for other conditions. Further studies are required to determine whether the finding in mice also works well in human. And, this is the limitation in our study.

ACKNOWLEDGMENTS

This research was supported partially by the National Natural Science Foundation of China (81501880 to Yunpeng Zhao); this research was also supported by the Natural Science Foundation of Shandong Province (BS2014YY048 to Yunpeng Zhao) and Scientific Research foundation of Qilu Hospital (26010175616073 to Yunpeng Zhao).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. None.

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