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Sauchinone inhibits IL-1β induced catabolism and hypertrophy in mouse chondrocytes to attenuate osteoarthritis via Nrf2/HO-1 and NF-κB pathways

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ABSTRACT

Background: Osteoarthritis (OA) is a common degenerative joint disease for which currently no anti-inflammatory therapy is available. Sauchinone (SAU), a key bioactive compound derived from *Saururus chinensis*, which has shown remarkable anti-inflammatory effects.

Methods: To evaluate the effect of SAU on OA progression, mouse chondrocytes were pretreated with SAU and subsequently stimulated with interleukin (IL)-1β. We found that SAU reduced the production of pro-inflammatory cytokines, such as nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α), and IL-6. SAU also inhibited the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) at both the gene and protein level. Moreover, SAU promoted the expression of aggrecan, while inhibiting the expression of catabolic factors, such as matrix metalloproteinases (MMPs) and thrombospondin motifs 5 (ADAMTS-5) in mouse chondrocytes. Col X, vascular endothelial growth factor-A (VEGF)-A, and Runx2, major markers of hypertrophic chondrocytes, were markedly elevated following IL-1β stimulation, and were reduced by SAU treatment while having the opposite effect on Col II. Mechanistically, we found that SAU inhibited nuclear factor kappa B (NF-κB) and activated the Nrf2/HO-1 pathway. The beneficial effects of SAU were also observed in vivo using a mouse OA model.

Conclusions: Our findings indicate that SAU may be a potential novel therapeutic for the treatment of OA.

1. Introduction

Osteoarthritis (OA) is a chronic, degenerative inflammatory disease resulting from wear of articular cartilage over time. OA induces pain, joint stiffness and dysfunction and is a socioeconomic burden for a significant proportion of the human population [1]. Although several mechanisms have been identified that contribute to OA development, the pathological etiology of OA is still unknown, and there currently no treatments are available that prevent disease progression [36]. Currently prescribed pharmaceutical agents such as non-steroidal anti-inflammatory drugs (NSAIDS), bisphosphonates and intra-articular hyaluronan injections only ameliorate clinical symptoms and have been related to detrimental side effects [37]. Therefore, it is essential to identify safe and effective drugs, which can reverse the progression of OA with minimal or no side effects.

The inflammatory OA environment alters chondrocyte phenotype and extracellular matrix (ECM) hemostasis, which aggravates cartilage destruction and promotes OA progression [43–45]. Accumulating evidence has indicated that inflammatory cytokines, such as interleukin (IL)-1β, contribute to disease development and progression through modulation of chondrocyte metabolism [5]. In OA, IL-1β is overproduced and secreted by activated synoviocytes and stimulates chondrocyte metabolism [8]. IL-1β induces production of catabolic factors such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) in chondrocytes to trigger cartilage destruction [9]. In addition, IL-1β triggers hypertrophic-like conversion of chondrocytes, thereby reducing the level of chrondocytes available to replenish collagen in the articular cartilage [12]. Thus, targeting IL-1β-induced inflammation may be an effective method to treat and prevent OA.

Sauchinone (SAU), a bioactive compound extracted from the root of the perineal herb *Saururus chinensis*, has gained increasing interest as a therapeutic agent due to its diverse pharmacological effects including anti-inflammatory activity. SAU has been shown protect against

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vascular inflammation in human umbilical vein endothelial cells [41] and inhibit elevation of pro-inflammatory factors inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNFα), and cyclooxygenase-2 (COX-2) in macrophages [42]. Moreover, SAU has shown to inhibit the osteoclast differentiation and bone resorption [27].

In the present study, we investigated the effect of SAU on IL-1β-induced inflammation in mouse chondrocytes and an animal model of OA.

2. Materials and methods

2.1. Reagents

SAU (purity > 98%) was purchased from Solarbio (Beijing, China). Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamo, Japan). Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). ELISA kits for the detection of mouse PGE2, IL-6, and TNF-α were purchased from R&D systems (Minneapolis, MN, USA). Primary antibodies directed against GAPDH, Lamin B, Type II Collagen, COX-2, iNOS, MMP-3, MMP-13, ADAMTS-5, aggrecan, Nrf2, HO-1, P65, p-IκBα, and Collagen X were purchased from Abcam (Cambridge, MA, USA). VEGF-A, aiti-RUNX-2, horseradish peroxidase (HRP)-conjugated goat anti-rabbit, and goat anti-mouse IgG were obtained from Bioworld (OH, USA). Alexa Fluor®488-labeled and Alexa Fluor®594-labeled goat anti-rabbit IgG (H + L) secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). The nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Beyotime (Shanghai, China). Dulbecco's modified Eagle's Medium (DMEM)/F12, fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Healthcare Life Sciences (Hyclone, Logan, UT, USA). TRIzol regent was purchased from Invitrogen (Carlsbad, CA, USA). QuantiTect Reverse Transcription kit was purchased from Qiagen (Valencia, CA, USA), and SYBR Green Master Mix was purchased from Bio-Rad Laboratories (CA, USA).

2.2. Isolation and culture of chondrocytes

Ten 10-day old C57BL/6 mice (5 males and 5 females) were euthanized using an overdose of sodium pentobarbital, and cartilage was removed from the knee and hip joints. Cartilage was then minced and washed with phosphate-buffered saline (PBS), containing penicillin-streptomycin and centrifuged at 1000 rpm at 37 °C for 3 min. A total of 6–10 mL of 0.2% type II collagenase was added to the tissue and digestion was performed for 4–6 h in the incubator maintained at 5% CO_2 at 37 °C. Detached cells were collected, centrifuged at 1000 RPM for 3 at 37 °C minutes, and transferred to a culture flask and incubated (37 °C, 5% CO_2) for 24 h. Once the culture reached 80% to 90% confluency, cells were harvested using 0.25% Trypsin-EDTA (Gibco, Invitrogen). Then, cells were replated into 10 cm culture plates at a density of 2×10^5 cells/mL in DMEM/F12 supplemented with 10% FBS and 1% antibiotic at 37 °C and 5% CO_2, and cell morphology and adherence were evaluated.

2.3. Cell viability

Cell viability was determined using the cell counting kit-8 (CCK-8; Dojindo Co, Kumamoto, Japan), according to the manufacturer's guidelines. In brief, P3 chondrocytes were seeded in 96-well plates (5×10^4 cell/cm^2). When cells reached a confluency of 90%–95%, culture medium was replaced with medium containing 0, 1, 3, 10, 30, 50, or 100 μM SAU, and cells were cultured for another 24 h or 48 h. Then, 10 μL of CCK-8 solution was added to each well and after 2 h, the absorbance was measured at a wavelength of 450 nm.

2.4. ELISA

ELISA kits (R&D Systems, Minneapolis, MN USA) were used according to the manufacturer's instruction.

2.5. Griess reaction

The production of nitric oxide in the culture medium was indirectly detected by the Griess reaction as previously described [21]. Briefly, Griess reagent was prepared, added to the culture medium, and incubated for 10 min. The absorbance was read at 543 nm.

2.6. Western blot analysis

Total proteins were isolated from cultured chrondocytes using RIPA lysis buffer with 1 mM PMSF (Phenylmethanesulfonyl fluoride). The samples were incubated on ice for 10 min, and centrifuged for 15 min at 12000 rpm and 4 °C. Protein concentration was determined using the BCA protein assay kit (Beyotime). A total of 40 ng proteins was loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and separated by electrophoresis. Then, proteins were transferred to PVDF membranes (Bio-Rad, USA). Following the transfer, membranes were blocked with 5% nonfat milk for 2 h and incubated overnight with primary antibodies directed against aggrecan (1:1000), Col X (1:500), Runx-2 (1:500), VEGF-A (1:500), HO-1(1:500), ADAMTS-5 (1:1000), NRF-2 (1:1000), COX-2 (1:1000), GADPH (1:5000), IκBα (1:1000), iNOS (1:1000), MMP-3 (1:1000), MMP13 (1:1000), Lamin B (1:1000), p65 (1:1000), GADPH (1:5000), and/or p-IκB (1:500) at 4 °C. Next, membranes were incubated with appropriate enzyme-linked secondary antibodies for 2 h at room temperature. To visualize immunoblots, enhanced chemiluminescence (ECL) solution was used according to the manufacturer's instructions.

2.7. Immunofluorescence analysis

Chondrocytes were fixed with 4% paraformaldehyde for 10 min, treated with 0.5%–2% Triton X-100 for 10 min, blocked with 10% Lowlenthal serum for an additional 60 min, and transferred to a wet box. Chondrocytes were incubated overnight with primary antibodies directed against collagen II (1:200), MMP-13 (1:200), p65 (1:200), and/or Nrf2 (1:200). Cells were then washed with PBS, and incubated with Alexa Fluor®488 conjugated or Alexa Fluor®594 conjugated secondary antibodies (1:400) in the dark for 1 h at room temperature. Finally, nuclei were stained with DAPI. Anti-fluorescence-quenching agent was used to mount the cover slip, and nail enamel was applied to seal the slides. The slides were preserved at 4 °C.

2.8. RNA isolation and quantitative real-time polymerase chain reaction

Chondrocytes were seeded in DMEM/F-12 in 6-well plates at a density of 3×10^5 cells/ml and incubated for 24 h. After stimulation with IL-1β and SAU at various concentrations (0, 1, 3, and 10 μM), total RNA was isolated from the monolayer of cultured chondrocytes using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 2000). The quality and purity of RNA was determined using the A260/A280 ratio. First-strand cDNA was synthesized using 1000 ng of total RNA and a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using the CFX96 Real-TimePCR System (Bio-Rad Laboratories, CA, USA) at the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The reaction was performed in a total volume of 10 μl (4.5 μl diluted cDNA, 0.25 μl forward primer, 0.25 μl reverse primer, and 5 μl SYBR Green Master Mix). Target mRNA levels were normalized to the GAPDH level, which was used as a control. Data were analyzed using the $2^{-\Delta\Delta CT}$ method [32].