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The protective impact of Trans-Cinnamaldehyde (TCA) against the IL-1β induced inflammation in *in vitro* osteoarthritis model by regulating PI3K/AKT pathways

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Abstract

Introduction. Osteoarthritis (OA) is a severe joint degeneration disease in elderly people described by the advanced degradation of articular cartilage, which ultimately leads to chronic pain. Trans-cinnamaldehyde (TCA) exerted its anti-inflammatory function in numerous disease syndromes; however, its role in the pathogenesis of OA remains unknown. The current research aimed to explore the potential protective impact of TCA in the progression of osteoarthritis *in vitro*.

Material and methods. Human knee articular chondrocytes were treated with 10 ng/ml IL-1β alone for 24 h or in a combination in a pretreatment with TCA at different concentrations (2, 5, 10 μg/mL, 24 h). The viability and cell apoptosis were determined by CCK-8 assay and flow cytometry methods. The protein levels of IL-8, PGE2, and TNF-α and the levels of phosphorylated AKT and PI3K were evaluated using ELISA assay. Moreover, RT-qPCR was used to measure the relative mRNA expression of MMP-13, iNOS, COX-2, and ADAMTS-5 in IL-1β-induced chondrocytes.

Results. Our results revealed that the treatment with TCA had no effect on chondrocytes' proliferation and apoptosis. Moreover, the protein levels of IL-8, TNF-α, and PGE2 were considerably reduced in IL-1β-induced chondrocytes treated with different concentrations of TCA. Furthermore, the mRNA expression of MMP-13, iNOS, COX-2, and ADAMTS-5 and the phosphorylation of AKT and PI3K were markedly reduced in IL-1β--induced chondrocytes with the increase in the concentration of TCA.

Conclusions. Trans-cinnamaldehyde inhibited the inflammation induced by IL-1β in chondrocytes through the PI3K/AKT pathway, which suggests that TCA might serve as a potential therapeutic agent for osteoarthritis treatment. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 4, 264–271)*

Key words: human articular chondrocytes; IL-1β; osteoarthritis model; trans-cinnamaldehyde; PI3K/AKT pathway; RT-qPCR; ELISA

Introduction

Osteoarthritis (OA) is a progressive syndrome described by disability and prolonged pain in the joints found most often in elderly people [1]. The main pathological manifestations during the development of OA include inflammation of the synovium, subchondral bone rebuilding, articular cartilage destruction [2, 3]. OA is closely related with many factors, including abnormal metabolism, obesity, joint malformation, aging, and osteoporosis [4, 5]. However, very little research has been done in exploring the molecular mechanism of OA pathogenesis. A previous study revealed that the increased IL-6, NO, TNF-α,

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and IL-1β were observed in serum and synovial fluid of osteoarthritis patients [6]. These findings provided indications that inflammation is an important factor in OA pathogenesis.

Cinnamomum cassia (C. cassia) is an herb previously used for the treatment of cancers, diabetes, inflammatory syndromes, ischemia, and dyspepsia all over the world [7, 8]. Trans-cinnamaldehyde (TCA), a natural product, extracted from the bark of C. cassia, exerts its important function in treating ischemia, arrhythmia, inflammatory syndromes and for the cold pathogenic diseases including pain, fever, sweating and headaches [9]. TCA showed its anti-inflammatory effect in macrophages/monocytes, endothelial cells and in aging rats [10–12]. TCA was shown to inhibit the neuro-inflammation in LPS-induced microglia BV2 cells system [13]. A study in mice revealed that TCA suppressed the collagen-induced inflammation *in vivo* model of arthritis [14]. Another study revealed that TCA inhibited the expression of inflammatory factors such as COX-2 and iNOS *via* activation of NF-κB pathway in the aging process [12]. Furthermore, cinnamic aldehyde suppressed the expressions of MMP-13, TNF-α, IL-6 and ADAMTS in LPS-induced osteoarthritis chondrocytes [15]. Moreover, TCA repressed the induction of COX-2 and iNOS in dopaminergic injury induced by 6-hydroxydopamine in both animal and cellular models [16]. These data demonstrated that TCA might serve as an anti-inflammatory agent and function in the activation of cells related with inflammatory diseases. However, the underlying mechanism of TCA in OA pathogenesis is still unclear.

One of the effects of the IL-1β action is the regulation of matrix metalloproteinases (MMPs), enzymes capable of degrading the extracellular protease enzymes including ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin motifs) and ECM proteins, laminin and collagen [17, 18]. Hence, IL-1β is associated with cartilage destruction. Previous research showed that IL-1β was markedly enhanced in the synovium fluid of OA patients [19]. Furthermore, it was reported recently that the secretions of inflammatory modulators such as nitric oxide (NO) and prostaglandin E2 (PGE2) were significantly elevated by IL-1β; hence, suggesting that targeting the inhibitors of these inflammatory modulators could be a therapeutic strategy for the treatment of OA [20].

Phosphatidylinositol 3-kinase/Protein kinase 3 (PI3K/AKT) signaling exerts their function in the modulation of numerous biological processes, including cell proliferation, differentiation, apoptosis and invasion [21]. Recently, PI3K/AKT signaling was also revealed as a critical pathway in association with inflammatory response [22]. The PI3K/AKT signaling has been identified to be activated in various human cancers [23]. However, the dual roles of PI3K in diseases depend on the various cellular responses including cell survival, trafficking of intracellular organelles in different cell types, remodeling of cytoskeletal proteins and others [24, 25]. On one hand, PI3K/AKT signaling pathway was found to be negatively regulated in the LPS-induced inflammation in RAW 264.7 macrophage cell line [26]. On the other hand, PI3K positively regulates the production of the proinflammatory cytokine IL-12 in LPS-induced human macrophages and dendritic cells (DCs) [27]. It was observed that PI3K/AKT activation takes place in the course of differentiation and proliferation of rat osteoblasts [28]. However, the exact molecular mechanism of PI3K/AKT signaling in OA still remains unclear. In the current research, we explored the anti-inflammatory effects of TCA in an *in vitro* model of OA.

Material and methods

Cell culture and treatments. The Human Knee Articular Chondrocytes were obtained from Lonza Pharma & Biotech, Levallois Perret, France. The cells were obtained and suspended in DMEM/F12 medium (Thermo Fisher, Parsippany, NJ, USA) containing 10% Fetal Bovine Serum (FBS; Biocompare, Toronto, ON, Canada), penicillin (100 U/ml) and streptomycin (100 μg/ml). Six-well plates were used to seed cells at a density 2×10^5 cells/ml and incubated in an incubator at 37°C with 5% CO_2. After every three 3 days the medium was substituted. The cells at 85–90% confluence were passaged using trypsin-EDTA solution (0.25%). The first passage of chondrocytes were collected after 14 days and we used only first two passage for the further experiments to avoid loss of phenotype. To explore the anti-inflammatory impacts of trans-cinnamaldehyde (TCA; Sigma Aldrich, St. Louis, MO, USA), cells were treated with 10 ng/ml IL-1β (Invivo Gen Pak Shek Kok, Hong Kong, China) alone or in a combination in a pretreatment with TCA at different concentrations (2, 5, 10 μg/mL). For the medium change, the control group was left untreated. Cells were collected after 24 h of incubation. To explore the PI3K/AKT signaling activation induced by IL-1β, 2 h of duration was set for the stimulation of cells with IL-1β. Besides, to examine the functional changes including inflammatory mediators, the duration for the stimulation of IL-1β was extended to 24 h.

CCK-8 assay. CCK-8 assay (Abcam, Cambridge, MA, USA) was used to measure the cytotoxic effects of TCA on chondrocytes following manufacturer's protocols. 96 well plates at a density 5×10^3 cells per well were used to seed chondrocytes and then treated with different concentrations (2, 5, 10 μg/mL) of TCA for 24 h. The cells were washed with PBS

rinsed twice followed by adding of CCK-8 solution (10 μl) into each well and incubated again for 3 h at 37° C. The absorbance was examined at 460 nm using a microplate reader (Thermo Fisher). All trials were repetitive three times.

Flow cytometry. Human Knee Articular Chondrocytes were rinsed twice with 10% phosphate-buffered saline (PBS), and a concentration of 1×10^5 cells/ml was adjusted by using a binding buffer. 500 μl of 1X Annexin V Binding Buffer solution purchased from Abcam (1×10^5 cells) was added into the culture tube followed by the staining with 1 μl Annexin-V-FITC and 1 μl propidium iodide in the dark at room temperature for 5 min. The cells were analyzed using the FACScan flow cytometer (LabX, Washington, VA, USA).

ELISA assay. Six-well plate at a density 1×10^6 cells/well were used to seed chondrocytes in order to measure the levels of secreted PGE2, IL-8, and TNF-α. When the cells reached a confluence 80%, then they were pretreated with a sample of trans-cinnamaldehyde (2, 5, 10 μg/mL) for 2 h alone, followed by the treatment with 10 ng/ml IL-1β for 24 h. The cultured media were harvested, and the SensoLyte ELISA kit (AnaSpec, Fremont, CA, USA) was used to detect the concentration of TNF-α, IL-8, and PGE2 following the manufacturer's protocol. The absorbance was examined at 460 nm using a microplate reader (Thermo Fisher). Moreover, the phosphorylation levels of PI3K and AKT proteins were measured by ELISA assay kit (AnaSpec, Fremont, CA, USA). More specifically, a standard working fluid (100 μl) was added to the hole of the corresponding plate and incubated at 37° C for 90 min. A bioionized antibody working fluid (100 μl) was added instantly and re-incubated for 60 min at 37° C. Then liquid in the plate was discarded, and the plate was rinsed 3 times, followed by the addition of HRP enzyme binding fluid (100 μl) and incubated for 30 min. Finally, a microplate reader (Thermo Fisher) was used to measure the absorbance at 450 nm.

qRT-PCR The total RNA was extracted from chondrocytes incubated with IL-1β (10 ng/ml) and different concentrations of TCA by using TRIzol reagent (Thermo Fisher). PrimeScript RT Master Mix kit (Takara Bio, Geumchoen-gu, Seoul, South Korea) was used to reverse transcribe RNA into cDNA. The primers for iNOS, ADAMTS-5, COX-2, and MMP-13 were amplified in a Step One Plus real Time PCR system by using SYBR Premix Ex Taq II kit (Thermo Fisher). The following parameters were used to perform RT-qPCR: 10 min at 95° C, 95° C 40 cycles of 15 sec and 1 min at 60° C. The threshold standards of each cycle were gathered and stabilized the level of mRNA to GAPDH mRNA level. The primers used are given in the Table 1. The results of PCR were calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze all the data that were presented as a mean ± standard deviation (SD). All experiments were repeated for three times. Graphpad Prism 7.0 (GraphPad, San Diego, CA, USA) was used for the statistical analysis. One way analysis of variance (ANOVA) and Student's t-test were used for the comparison among two more groups. The value of p less than 0.05 was taken as statistically different (**) while p value less than 0.01 was taken as notably different (***).

Table 1. Primer sequences

Primers		Sequence
iNOS	Forward	ACCAACTGACGGGAGATGAG
iNOS	Reverse	GTTGCCATTGTTGGTGGAGT
ADAMTS-5	Forward	TGTCACTAGTCATGGCAGCA
ADAMTS-5	Reverse	CAGAAGGCCTTTGGGAGAGA
COX-2	Forward	GAGAGATGTATCCTCCCACAGTCA
COX-2	Reverse	GACCAGGCACCAGACCAAAG
MMP-13	Forward	AGGCCTTCAGAAAAGCCTTC
MMP-13	Reverse	GAGCTGCTTGTCCAGGTTTC
β-actin	Forward	CACCATGTACCCTGGCATTG
β-actin	Reverse	CCTGCTTGCTGATCCACATC

Results

Impact of trans-cinnamaldehyde on the chondrocyte viability and apoptosis

To investigate the impact of TCA in osteoarthritis *in vitro* model, we treated chondrocytes with different concentrations (2, 5, 10 μg/mL) of TCA for 24 h. The cell viability and rate of cell apoptosis were determined by using CCK-8 assay and flow cytometry, respectively. Our findings revealed that TCA had no significant impact on the viability of chondrocytes and the rate of cell apoptosis of chondrocytes, even with the highest concentration of TCA of 10 μg/mL (Fig. 1A, B). Thus, no cytotoxicity of TCA was observed in the cultured human chondrocytes.

Trans-cinnamaldehyde (suppressed IL-1β-induced TNF-α, IL-8, and PGE2 secretion by chondrocytes

The levels of IL-8 and TNF-α were assessed by using ELISA assay, and our findings revealed that the IL-1β expressively augmented the level of IL-8 and TNF-α in the conditioned medium of chondrocytes compared to that of the control group. However, an increase in the concentration of TCA markedly inhibited the levels of these inflammatory cytokines (Fig. 2A, B). Moreover, IL-1β significantly enhanced PGE2 level in chondrocytes' medium, while TCA considerably

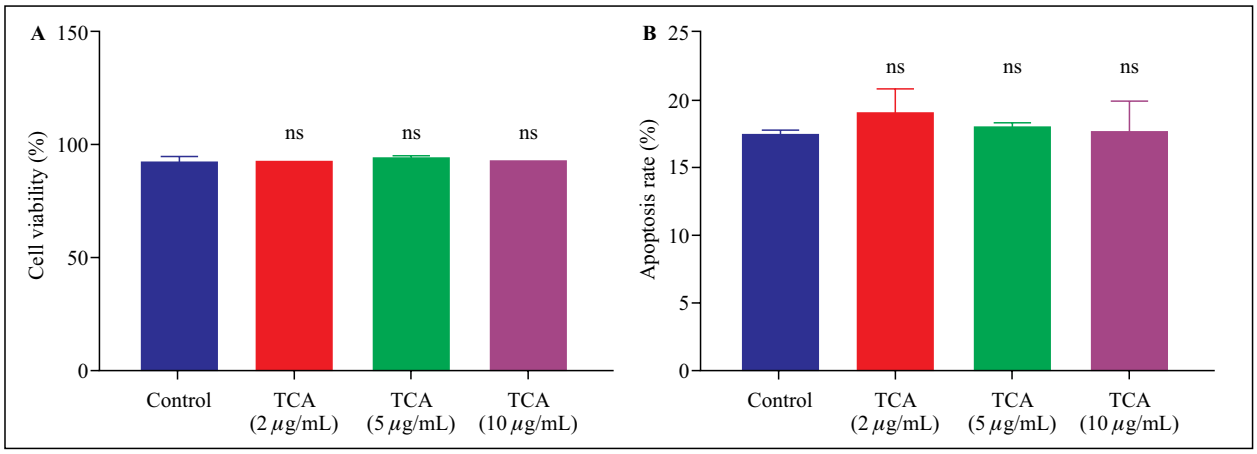

Figure 1. Impact of trans-cinnamaldehyde (TCA) on the chondrocyte viability (**A**) and apoptosis (**B**) that were measured as described in Methods. Bars and whiskers represent mean ± standard deviation (SD). ns, no significant difference compared with control.

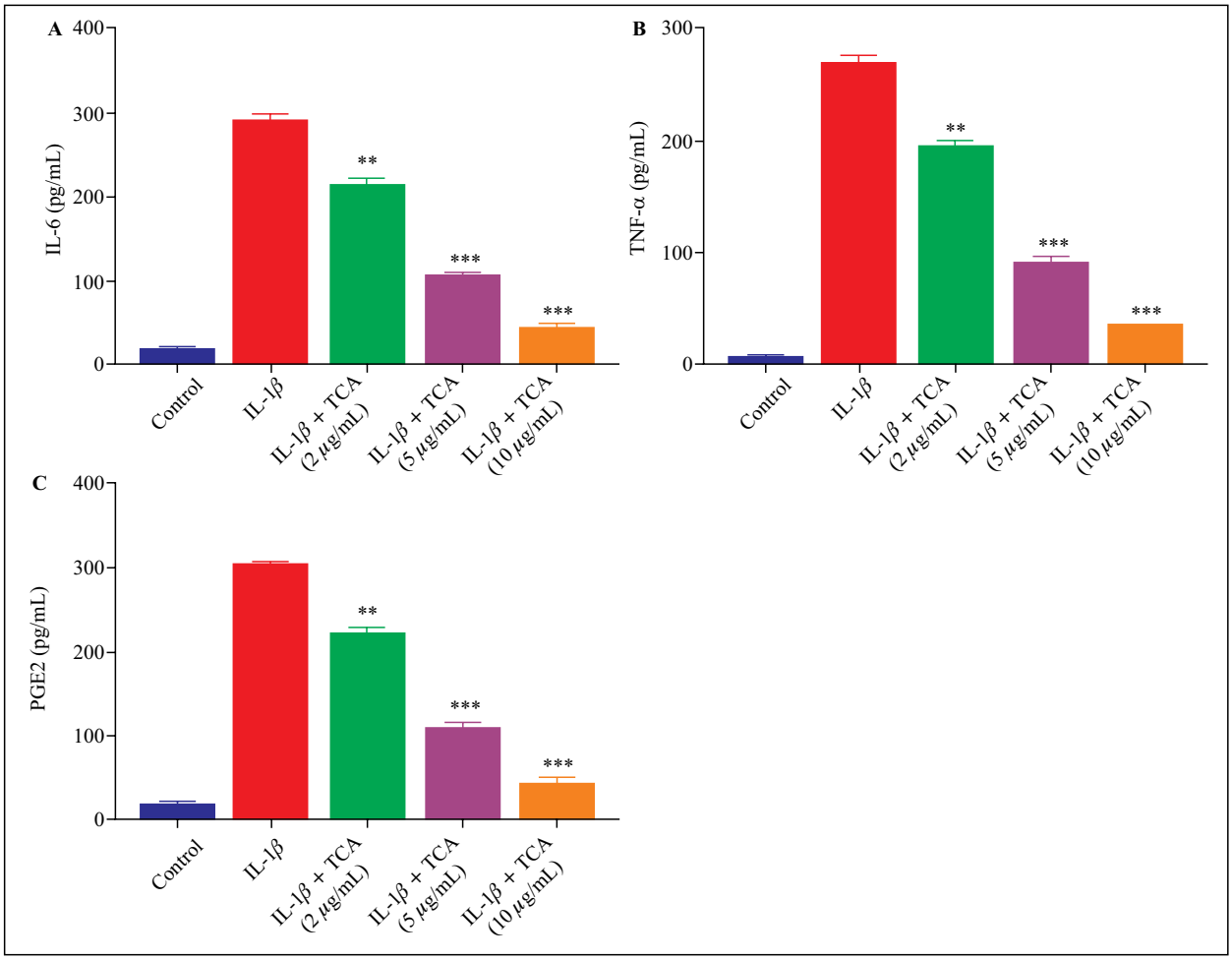

Figure 2. Trans-cinnamaldehyde suppressed IL-1β-induced IL-6, TNF-α, and PGE2 in chondrocytes: The concentrations of TNF-α (**A**), IL-6 (**B**), and PGE2 (**C**) were determined in the cultured media of chondrocytes treated with or without different concentrations of TCA as described in Methods. **p < 0.05, ***p < 0.01 TCA treatment group *vs*. IL-1β group.

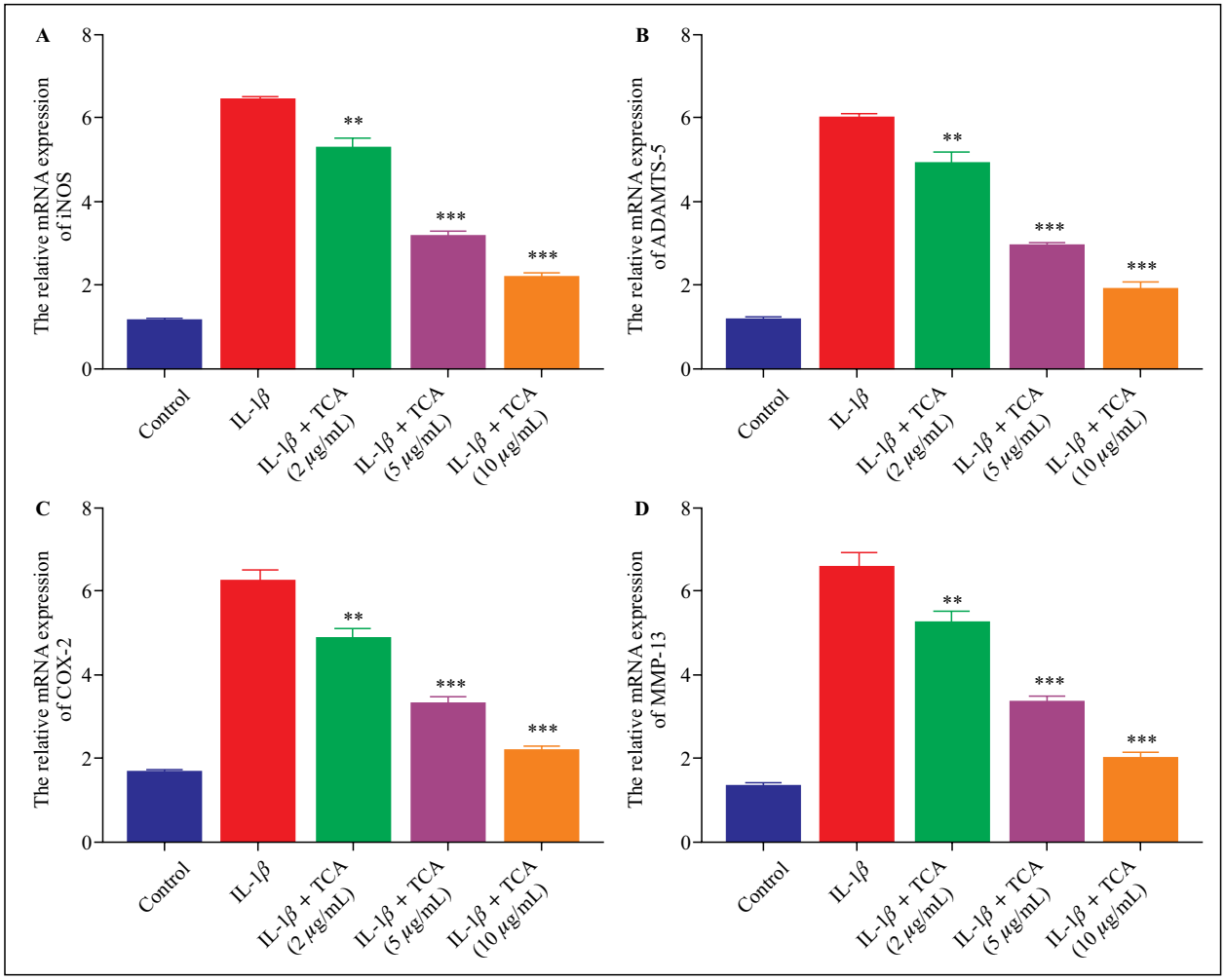

Figure 3. Impact of trans-cinnamaldehyde on iNOS, ADAMTS-5, COX2 and MMP-13 expression in human chondrocytes *in vitro*. The mRNA expressions of iNOS (**A**), ADAMTS-5 (**B**), COX-2 (**C**), and MMP-13 (**D**) were determined in chondrocytes treated IL-1β with or without different concentrations of TCA by using qRT-PCR. **p <0.05, ***p < 0.01 TCA treatment group *vs*. IL-1β group.

reduced the levels of PGE2 in a dose-dependent manner (Fig. 2C).

Effect of trans-cinnamaldehyde on iNOS, ADAMTS-5, COX2 and MMP-13 relative mRNA levels in IL-1β–induced chondrocytes

By using RT-qPCR, we found that IL-1β evidently enhanced mRNA iNOS level in chondrocytes compared to the control group. Moreover, iNOS expression was significantly decreased when chondrocytes were treated with increasing concentrations of TCA (Fig. 3A). Furthermore, the relative mRNA levels of MMP-13, ADAMTS-5, and COX-2 were considerably enhanced in IL-1β-induced chondrocytes as compared to the control group while TCA markedly decreased the mRNA levels of MMP-13, ADAMTS-5, and COX-2 in IL-1β-induced chondrocytes (Fig. 3B–D).

Trans-cinnamaldehyde inhibited the PI3K/AKT pathway activation in IL-1 β-induced chondrocytes

Further, we examined the impact of TCA on the phosphorylation of PI3K/AKT signaling pathway and results of the ELISA assay showed that the phosphorylation of PI3K/AKT signaling pathway components was markedly enhanced in chondrocytes treated with IL-1β alone compared to that of the control group. Besides, increasing concentration of TCA significantly decreased the phosphorylation of the PI3K/AKT signaling pathway in chondrocytes (Fig. 4A–B).

Discussion

Osteoarthritis is a severe joint ailment that causes loss of articular cartilage, functional disabilities, and joint pain due to various factors [1]. TCA has been

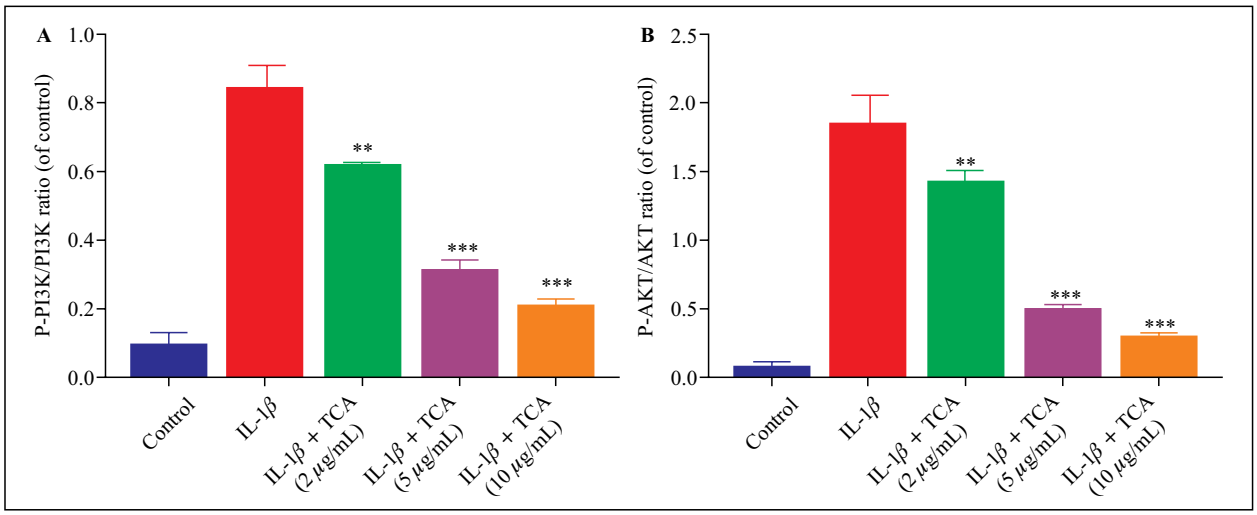

Figure 4. Trans-cinnamaldehyde inhibited the ratio of PI3K/AKT signaling pathway components. The relative mRNA expression of p-PI3K (**A**), and p-AKT (**B**) were determined in chondrocytes treated with or without different concentrations of TCA by using RT-qPCR. $^{**}p < 0.05$, $^{***}p < 0.01$ TCA treatment group *vs* IL-1β group.

reported to suppress the inflammatory injury in *in vivo* model of neuro-inflammation and LPS-activated BV2 microglia [13]. Moreover, TCA exerts its anti-inflammatory impact [29], including inhibition of PGE2, NO, and IL-1β synthesis in the mice challenged with lipopolysaccharide [30]. Another study indicated that TCA decreased the level of MMPs (MMP-3, MMP-1, MMP-13) and ADAMTS-5 in human chondrocytes and in SW1353 cells in *in vitro* OA model [31]. However, whether TCA exerts its function on the expression of ADAMTS and MMPs through PI3K/AKT signaling has not yet been elucidated. In this paper, we developed OA *in vitro* model and examined the potential protective effects of TCA in IL-1β-induced inflammation in human knee articular chondrocyte. Our findings revealed that TCA exerts its protective impact against inflammation induced by IL-1β through suppressing PI3K/AKT signaling pathway.

The results of former studies showed that IL-1β regulates MMPs' induction in human chondrocytes [32]. IL-1β was unveiled to modulate cartilage destruction and cartilage matrix degradation in osteoarthritis pathogenesis [33]. In cultured human osteoarthritis chondrocytes, it enhanced the level of inflammatory modulators such as NO and PGE2 and the secretion of IL-6 and TNF-α, leading to the aggravation of inflammation [34]. The iNOS present in chondrocytes is responsible for the excessive expression of NO. It has been previously reported that NO exerts its catabolic effect in OA pathogenesis by decreasing the production of collagen and proteoglycans, resulting in their enhanced degradation [35].

Previous studies showed that urolithin A significantly alleviated the pancreatic ductal adenocarcinoma (PDAC) progression in xenograft mice through the down-regulation of PI3K/AKT signaling [36]. Another study demonstrated that dexamethasone reduced the ATDC5 cell chondrogenesis induced by insulin *via* inactivation of the PI3K/AKT pathway [37]. A recent study revealed that the activation of PI3K/AKT pathway notably contributed to the generation of COX-2 and MMPs in LPS-induced inflammation in Acute Lung Injury (ALI) model [38]. Inactivation of AKT facilitated chondrocyte apoptosis induced by dexamethasone [39]. PI3K/AKT pathway enhanced the autophagy of articular chondrocytes and inhibited the inflammatory reaction in OA *in vivo* rat model [40]. However, whether these events play a role in the pathogenesis of OA through the activation of PI3K/AKT signaling pathway still needs unveiling. Our current study demonstrated that IL-1β expressively enhanced the phosphorylation of PI3K and AKT in chondrocytes. On the other hand, adding increasing concentrations of TCA to the human chondrocytes incubated simultaneously with IL-1β dramatically inhibited the phosphorylation of PI3K and AKT in chondrocytes, implying that the TCA pretreatment could alleviate the levels of inflammatory mediators in a concentration-dependent manner and have impact on the gene expression of COX-2, PGE2, MMP-13, IL-6, TNF-α and ADAMTS-5.

PGE2 is considered as an essential inflammatory mediator synthesized by COX-2. Clinical studies demonstrated that antagonists or inhibitors of COX-2 exert its function in the protection of OA cartilage by

inhibiting inflammation both *in vivo* and *in vitro* [41]. Moreover, the inhibitors of PGE2 may assist as therapeutic targets for OA treatment to counteract the anti-anabolic effect of PGE2 observed in human articular cartilage *in vitro* [42]. Our findings demonstrated that TCA expressively reduced the levels of PGE2 and COX-2 mRNA, suggesting that TCA might serve as a therapeutic drug for OA treatment. Moreover, ADAMTS-5 and MMP-3 are also crucial proteins in OA. ADAMTS-5 is involved in the cleavage of aggrecan in the course of OA progression [43]. MMP-13 has been found to be involved in the decomposition of the extracellular matrix (ECM) in various biological processes; however, the excess amount of MMP-13 leads to excess ECM destruction of OA [44]. The current study pointed out that increasing concentration of TCA expressively inhibited ADAMTS-5 and MMP-13 mRNA expression in chondrocytes. Our data revealed that TCA may protect the degradation of cartilage extracellular matrix by suppressing the over-expression of ADAMTS-5 and MMP-13 in chondrocytes stimulated by IL-1β.

In conclusion, we have demonstrated that trans-cinnamaldehyde could inhibit the inflammation induced by IL-1β in chondrocytes *in vitro via* regulating the PI3K/AKT pathway suggesting that TCA might be used as the potential drug for OA treatment.

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