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Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages

Shengqian Q Wu[a,b], **Miguel Otero**[c], **Frank M Unger**[a], **Mary B Goldring**[c], **Ampai Phrutivorapongkul**[d], **Catharina Chiari**[e], **Alexander Kolb**[e], **Helmut Viernstein**[a], and **Stefan Toegel**[a,c,e,*]

[a]Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, 1090 Vienna, Austria [b]Department of Pharmacology and Toxicology, University of Vienna, 1090 Vienna, Austria [c]Laboratory of Cartilage Biology, Research Division, Hospital for Special Surgery, Weill Cornell Medical College, New York, USA [d]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand [e]Department of Orthopedics, Medical University Vienna, 1090 Vienna, Austria

Abstract

Aim of the study—*Caesalpinia sappan* is a common remedy in Traditional Chinese Medicine and possesses diverse biological activities including anti-inflammatory properties. Osteoarthritis (OA) is a degenerative joint disease with an inflammatory component that drives the degradation of cartilage extracellular matrix. In order to provide a scientific basis for the applicability of *Caesalpinia sappan* in arthritic diseases, the present study aimed to assess the effects of an ethanolic *Caesalpinia sappan* extract (CSE) on human chondrocytes and macrophages.

Materials and Methods—Primary human chondrocytes were isolated from cartilage specimens of OA patients. Primary cells, SW1353 chondrocytes and THP-1 macrophages were serum-starved and pretreated with different concentrations of CSE prior to stimulation with 10 ng/ml of interleukin-1beta (IL-1ß) or lipopolysaccharide (LPS). Following viability tests, nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) were evaluated by Griess assay and ELISA, respectively. Using validated real-time PCR assays, mRNA levels of IL-1ß, TNF-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were quantified. SW1353 cells were cotransfected with a COX-2 luciferase reporter plasmid and nuclear factor-kappa-B (NF-κB) p50 and p65 expression vectors in the presence or absence of CSE.

Results—CSE dose-dependently inhibited the expression of pro-inflammatory cytokines IL-1ß and TNF-α in IL-1ß-stimulated chondrocytes and LPS-stimulated THP-1 macrophages. CSE further suppressed the synthesis of NO in primary OA chondrocytes by blocking iNOS mRNA expression. The inhibition of COX-2 transcription was found to be related with the CSE inhibition of the p65/p50-driven transactivation of the COX-2 promoter.

Conclusions—The present report is first to demonstrate the anti-inflammatory activity of CSE in an in vitro cell model of joint inflammation. CSE can effectively abrogate the IL-1ß-induced

*Corresponding author Stefan Toegel, Department of Orthopedics, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, AUSTRIA, Tel: 0043 1 40400 7653, Fax: 0043 1 40400 7641, stefan.toegel@meduniwien.ac.at.

over-expression of inflammatory mediators at the transcriptional level in human chondrocytes and macrophages, most likely by inhibiting NF-κB (p65/p50) signaling. Blockade of IL-1ß-induced NF-κB signaling and its downstream pro-inflammatory targets by CSE may be beneficial for reducing cartilage breakdown in arthritis.

Keywords

osteoarthritis; inflammation; chondrocytes; *Caesalpinia sappan*; cytokine; COX-2

1. Introduction

Osteoarthritis (OA) ranks among the major causes of physical disability of elderly patients, thus representing a critical factor in health economics. In contrast to rheumatoid arthritis, OA is conventionally not considered a classical inflammatory arthropathy, but thought to develop from chronic overuse or injury of the joint. However, evidence has accumulated that, besides mechanical and genetic factors, inflammatory processes within joint tissues contribute to the OA onset and progression. Chondrocytes, the unique cell component of articular cartilage, are embedded in a highly organized extracellular matrix (ECM), comprising collagen type II fibrils and proteoglycans, which confer to the cartilage structural rigidity and protective resiliency (Goldring and Marcu, 2009). In response to mechanical or biochemical stress, chondrocytes overexpress pro-inflammatory mediators such as interleukin-1beta (IL-1ß) and tumor necrosis factor-alpha (TNF-α) that will, in an autocrine/paracrine manner, stimulate their own production and induce the expression of matrix-degrading proteinases, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Goldring and Goldring, 2007; Bonnet and Walsh, 2005). Recently, it has been demonstrated that mediators such as prostaglandin E_2 (PGE_2) (Gosset et al., 2010) or nitric oxide (NO) (Tsuruda et al., 2004) play key roles in the induction of MMPs in an inflammatory context. In addition to the chondrocytes, macrophages that have infiltrated the OA synovium contribute to inflammation and matrix degradation in OA tissues (Benito et al., 2005; Bodenson et al., 2006). Therefore, inflammatory mediators represent potential targets for OA disease modification.

The mechanism of cartilage degradation in OA is known to be a multifactorial process. However, standard pharmacological interventions, such as the use of nonsteroidal anti-inflammatory drugs (NSAIDs), often act in a monomodal way that is frequently associated with significant adverse effects (Fendrick and Greenberg, 2009). Although considerable progress has been made in the development of novel strategies, such as the use of direct MMP inhibitors, no clinically effective inhibitor exists to date. Thus, novel, safe and effective anti-inflammatory agents are demanded for the therapy of arthritic diseases. Nutraceuticals and phytopharmaceuticals that usually contain a range of active compounds targeting multiple pathways could provide an alternative to conventional treatment of OA (Clutterbuck et al., 2009). A comprehensive review on current nutraceuticals for the management of OA has been published elsewhere (Ameye and Chee, 2009).

Sappan Lignum, the heartwood of *Caesalpinia sappan* L. (Leguminosae), is the source of a natural red dye. In traditional Chinese medicine, Sappan wood has sweet, salty, and neutral characteristics, and is associated with the heart, liver and spleen meridians. Traditionally, it is applied as an aqueous decoction and prescribed to invigorate the blood system, promote menstruation, reduce pain and swelling (Chinese Pharmacopoeia, 2010). Additionally, Sappan wood has been medicinally recommended due to its astringent or diuretic properties, as well as for certain skin diseases (Sireeratawong et al., 2010). Recently, it has also been listed in the 15th edition of the Japanese Pharmacopoeia (Japanese Pharmacopoeia, 2006).

Different extracts and active compounds isolated from Sappan lignum have been reported to possess diverse biological activities including antioxidative (Budami et al., 2003), antiinflammtory (Jeong et al., 2008), antibacterial (Xu and Lee, 2004) and anticonvulsive (Baek et al., 2000) effects. Brazilin, the major compound of Sappan Lignum, was reported to have anti-inflammatory (Hikino et al., 1977, Washiyama et al., 2009), antibacterial (Batubara et al., 2010), and antihepatotoxic effects (You et al., 2005). In addition, numerous other compounds from Sappan Lignum such as prostosappanins A-E or sappanchalcone have also been shown to display some of the different biological activities (Liu et al., 2009). To our knowledge, however, *Caesalpinia sappan* has not been evaluated in the context of OA, and the effects of *Caesalpinia sappan* derived isolates on OA chondrocytes and synovial macrophages are unknown.

The present study, therefore, aimed to elucidate the anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract (CSE) in lipopolysaccharide (LPS)-stimulated human macrophages and IL-1ß-stimulated human chondrocytes. Particular focus was on the inhibition of gene expression of proinflammatory factors such as IL-1ß and TNF-α, and on the interference with NO production and NF-κB-mediated COX-2 promoter activation.

2. Materials and Methods

2.1. Plant material and extraction

The heartwoods of *Caesalpinia sappan* were collected in June 2006 in San-Pa-Thong district, Chiang Mai province, Thailand, and identified by comparison with the voucher specimen (No. 87-1631) at the Herbarium Section, Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand. Powdered heartwood (30 g) was extracted with 95% ethanol (350 ml) for 24 h using a Soxhlet apparatus and the resulting extract was concentrated under vacuum to yield a solid extract (5.5g).

2.2. Characterization of the extract

Four batches of CSE were characterized by HPLC (ICS-3000, Dionex, USA) with a Nucleodur C-18 column (Macherey-Nagel, D) and a PDA-100 detector (Dionex) set at 280 nm. The eluent system consisted of a 45 min gradient program from 10% to 100% methanol (containing 2.5% acetic acid) at a flow rate of 0.8 ml/min. All 4 batches yielded congruent chromatograms, which were also comparable to that of Natural Red 24 (MP Biomedicals, USA, LOT #: R25179), a commercially available Sappan wood extract (Supplementary Figure 1A and 1B).

Brazilin was isolated from CSE using preparative HPLC (Shimadzu, Japan) with a reversed – phase C18 column (Polygoprep C18, 12μ, 25mm × 250mm, Austria) monitored at 280nm. The eluent system consisted of a 45 min gradient program from 10% to 100% methanol (containing 1.25% acetic acid) at a flow rate of 22 ml/min and a column temperature of 50°C. The fraction at a retention time of about 20 min was collected in repeated preparative HPLC separations. These fractions were further concentrated under reduced pressure and then freeze-dried to give a white compound which rapidly oxidized to a red-brown powder. In accordance with Chen et al. (Chen et al., 2008), the isolated compound was identified as brazilin ((6aS,11bR)-7,11b-Dihydro-6H-indeno[2,1-c]chromene-3,6a,9,10-tetrol) using ^{1}H NMR (Bruker Avance 500 instrument, Bruker, USA). The HPLC chromatogram of brazilin is shown in Supplementary Figure 1C.

2.2. Cell cultures

In accordance with the terms of the ethics committee of the Medical University Vienna (EK-Nr.: 081/2005) and following the guidelines of the Declaration of Helsinki and Tokyo,

primary human chondrocytes were enzymatically isolated from articular cartilage of OA patients undergoing knee replacement surgery following established protocols (Toegel et al., 2009; Toegel et al., 2010). Isolated chondrocytes were seeded at a density of $10^5/cm^2$ and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, A) containing 10% fetal calf serum (FCS; Biochrom AG, D) and 2 μl/ml gentamycin (Biochrom AG, D) in a humidified atmosphere of 5% CO_2/95% air at 37°C. For all assays, only freshly isolated and seeded cells without subculturing were used. The human chondrosarcoma cell line SW1353 (ATCC, USA) was cultured at a density of $10^4/cm^2$ under the same conditions as described above and passaged by incubation with 0.25% trypsin-EDTA. The human monocytic cell line THP-1 (ATCC, USA) was maintained in RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, and 104 U/ml penicillin (Gibco, A) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were differentiated into macrophages in the above medium containing 5 ng/ml phorbol 12-myristate 13-acetate (PMA) over 24 h. After washing once, cultures were changed to medium without PMA for 24 h and then used for further studies.

2.3. Cytotoxicity assay

The cytotoxicity of CSE was assessed in primary human chondrocytes and THP-1 cells using an MTT-based test (EZ4U, Biomedica, A) following the manufacturer's instructions. Briefly, 3×10^3 cells/well were seeded in quadruplicate in 96-well microplates (Iwaki, J) and cultured with various concentrations of CSE (0.625 to 40 μg/ml) for 3 days. The absorbance was recorded by a Spectrafluor Fluorometer (Tecan, A) at 450 nm with 620 nm as reference wave length.

2.4. Griess assay

Primary human OA chondrocytes (n=3 donors) were seeded in 96-well microplates and analyzed separately. Upon confluency, cells were preincubated with CSE at three concentrations (10 μg/ml, 5 μg/ml or 2.5 μg/ml) for 1 h prior to coincubation with 10 ng/ml IL-1ß (Miltenyi Biotec, D) for 24 h. The amount of accumulated nitrite derived from NO metabolism was determined in the cell culture supernatant by mixing with an equal volume of Griess reagent (1% sulphanilamide in 0.1 N HCl and 0.1% N-1-naphthylethylenediamine dihydrochloride in equal parts). Absorbance was measured at 544 nm with reference to 690 nm with a Spectrafluor Fluorometer. Nitrite concentrations were calculated using a $NaNO_2$ standard curve (0-100 μM in cell culture medium). A nitrite standard reference curve was established for each assay.

2.5. Quantitative real-time RT-PCR (RT-qPCR)

4×10^5/well primary human OA chondrocytes or 4×10^4/well SW1353 cells were seeded in 12-well microplates (Iwaki, J). Upon 90% confluency, medium was changed to DMEM containing 2% Insulin-Transferrin-Selenium (ITS; Gibco, A) and incubated overnight prior to treatment. Chondrocytes were preincubated with CSE (5, 10, or 20 μg/ml) or 10 μM dexamethasone (Sigma, A) for 1 h and then incubated for a further 6 h in the presence of 10 ng/ml IL-1ß. In a different set of experiment, cells were pretreated with 10 μg/ml Natural Red 24 (NR24). Cells without any treatment were used as control. THP-1 cells were seeded in 12-well microplates at 3×10^5 per well. After differentiation with PMA (see above), cells were preincubated with 5 μg/ml CSE for 1 h and then co-treated for an additional 2 h with 10 ng/ml LPS.

Total RNA was extracted from each sample using the NucleoSpin RNA II Kit (Macherey-Nagel, D) according to the manufacturer's instructions. To quantify and control for the integrity of the isolated RNA preparations, each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip prior to reverse transcription of equal RNA quantities into

cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, A). RNA integrity numbers (RIN) were found to range from 7.8 to 10 for all samples.

qPCR analyses of IL-1ß, TNF-α, iNOS and COX-2 mRNA were performed using primer sets described in Table 1. Primers for reference genes were used as previously described (Toegel et al., 2007). Amplification efficiencies of all primers were evaluated using dilution series of cDNA prepared from chondrocyte mRNA. All qPCR reactions were performed in 25 µl reaction mixtures containing 1 µl cDNA, 12.5 µl SensiMix SYBR Green Mix (GenXpress, A), 100 nM primers (Metabion, D), and nuclease-free water to 25 µl, and run in duplicate on an Mx3000P QPCR system (Stratagene, USA). Melting curves were generated to confirm a single gene-specific peak and no-template-controls were included in each run to control for contaminations.

The regulation of target genes was calculated as quantities relative to the untreated control group using the MxPro real-time QPCR software, considering both amplification efficiencies and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene. In preliminary experiments, the expression stabilities of 5 candidate reference genes (GAPDH, beta actin (ACTB), hypoxanthine phosphoribosyl-transferase I (HPRT1), succinate dehydrogenase complex, subunit A (SDHA), beta-2-microglobulin (B2M)) were determined under the experimental conditions described above. Then, the expression stabilities were evaluated using the geNorm software (Vandesomp et al., 2002) and GAPDH was selected as a stable reference gene under the experimental conditions of this study.

We declare that the current study has adhered to the minimal guidelines for the design and documentation of qPCR experiments as recently outlined by Bustin et al (Bustin et al., 2010). A qPCR checklist listing all relevant technical information has been provided for the reviewers to assess the technical adequacy of the used qPCR protocols (Supplementary Table 1).

2.6. TNF-α ELISA

4×10^5/well primary human OA chondrocytes from donors (n=2 donors) or 4×10^4/well SW1353 cells were seeded in 12-well microplates. Upon 90% confluency, culture medium was changed to DMEM containing 2% ITS and cultured overnight. After 1 h preincubation with 5 µg/ml CSE, the cells were treated with 10 ng/ml IL-1ß for an additional 48 h. Chondrocytes without any treatment were used as control. The TNF-α concentration in the cell culture supernatants was measured using the Human TNF alpha ELISA Ready-SET-Go! Kit (eBioscience, USA), as described by the manufacturer.

2.7. Transfections and reporter assays

The pXP2-*COX2*-Luc reporter construct, containing -170/+103 bp of the human *COX2* promoter, and the NF-kB p50 and p65 expression vectors were described previously (Grall et al., 2005). The sequences of all constructs were confirmed by DNA sequencing.

Transient transfection experiments were performed in SW1353 cells using LipofectAMINE PLUS Reagent (Invitrogen, A). Cells were seeded in 24-well plates at 10^4 cells/cm^2 in DMEM/F12 containing 10% FCS. Transfections were carried out in serum-free medium with a total of no more than 325 ng of plasmid DNA, including 300 ng of luciferase reporter construct and 25 ng per well of expression vector or empty backbone (control). Briefly, plasmid DNA was incubated for 15 min at room temperature with 6 µl Plus Reagent and 94 µl serum-free DMEM/F12. Following incubation, LipofectAMINE reagent (4 µl) in 96 µl serum-free medium was added to each reaction mixture and the incubation was continued for an additional 30 min at room temperature. Then, the transfection mixture was combined

with 300 μl of serum-free medium and the lipid-nucleic acid complex (500 μl) was added drop-wise to the cell monolayer yielding a total volume of 1 ml. After incubation for 1 h at 37°C, 1 ml of serum-free medium containing CSE was added to each well and incubation was continued for 23 h. Cell lysates were prepared using the Reporter Lysis Buffer (Promega, USA) and luciferase activities were determined by chemiluminescence assay using the LMaxII384 luminometer (Molecular Devices, USA) and the Luciferase Assay Substrate (Promega). Data are representative of two independent experiments performed in triplicate and expressed as fold-change *versus* unstimulated control.

2.8. Statistical Analysis

Data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). The Gaussian distribution of data was verified using the Kolmogorov-Smirnov test. Statistics were performed using one-way analysis of variance (ANOVA) with post hoc Tukey tests, cross-comparing all study groups (95% confidence interval). p-values < 0.05 were considered significant.

3. Results

3.1. Effect of CSE on cell viability

As shown in Figure 1, significant cytotoxicity of CSE was observed at concentrations higher than 40 μg/ml and 10 μg/ml in primary chondrocytes and THP-1 cells, respectively. The subsequent experiments were therefore performed using CSE concentrations not higher than 20 μg/ml for experiments carried out using human primary chondrocytes and at 5 μg/ml for treatment of THP-1 cells.

3.2. Effect of CSE on IL-1ß and TNF-α mRNA in THP-1 cells

In THP-1 macrophages, 10 ng/ml LPS induced an 11.9±0.6 fold and 60.2±7.2 fold upregulation of IL-1ß (Figure 2A) and TNF-α (Figure 2B) mRNA levels, respectively. Pretreatment with 5 μg/ml CSE significantly inhibited the upregulation of both cytokines in THP-1 macrophages. In the case of TNF-α, CSE pretreatment down-regulated the mRNA expression to levels comparable to control cells (Figure 2B).

3.3. Effect of CSE on IL-1ß and TNF-α expression in chondrocytes

As shown in Figure 3A, IL-1ß gene expression in SW1353 chondrocytes was upregulated by 45.3±2.5 fold following stimulation with 10 ng/ml recombinant IL-1ß. CSE, however, markedly suppressed the effect caused by IL-1ß in a concentration-dependent manner. At 10 and 20 μg/ml, CSE completely blocked the IL-1ß-induced IL-1ß mRNA level, re-establishing expression levels comparable to untreated cells. The TNF-α mRNA level (Figure 3B) was strongly increased 325±9 fold by 10 ng/ml IL-1ß, whereas pre-treatment with 10 or 20 μg/ml CSE significantly reduced this effect. In addition, comparable results regarding the impact of CSE on IL-1ß and TNF-α mRNA expression were obtained in primary chondrocytes (Table 2). Interestingly, the upregulation of IL-1ß and TNF-α mRNA expression caused by IL-1ß was considerable higher in primary chondrocytes than in SW1353 cells. Nevertheless, the stimulated expression of IL-1ß and TNF-α mRNA was clearly abrogated by CSE treatment in a dose-dependent manner. The validity of the assay was verified using 10 μM dexamethasone, a widely used anti-inflammatory glucocorticoid, as positive control. Dexamethasone substantially suppressed the IL-1ß-mediated upregulation of IL-1ß and TNF-α mRNA levels in primary chondrocytes isolated from 3 donors ($p<0.05$; data not shown).

Interestingly, also NR24 down-regulated TNF-α mRNA levels in IL-1ß stimulated primary chondrocytes (Supplementary Figure 2). Even more importantly, the inhibitory effects

mediated by NR24 were significantly less pronounced than those mediated by CSE ($p<0.05$). This fact might be explained by the lower concentration of brazilin found in NR24 as compared to CSE (Supplementary Figure 1).

The production of TNF-α was measured in the culture supernatants of chondrocytes using ELISA. As shown in Figure 4, 24 h treatment with IL-1ß (10 ng/ml) caused a 3.6 fold and 9.1 fold increase of TNF-α expression in SW1353 chondrocytes and primary human chondrocytes, respectively. Pretreatment of both cell types with 5 μg/ml CSE significantly suppressed the IL-1ß-induced TNF-α production. As such, 5 μg/ml CSE reduced the IL-1ß-induced TNF-α production in primary cells from 19.1±1.1 pg/ml to 5.4±3.8 pg/ml. In both SW1353 and primary cells, the TNF-α production was down-regulated to basal levels.

3.4. Effect of CSE on NO production and on iNOS expression

Nitrite, the stable metabolite of NO, was measured using the Griess assay in primary chondrocytes. Stimulation with IL-1ß resulted in a 16.3±4.9 fold increase of nitrite accumulation whereas pretreatment with CSE caused a concentration-dependent reduction of nitrite accumulation (Figure 5). 5 μg/ml and 10 μg/ml CSE significantly suppressed the IL-1ß-induced NO production to basal levels. At 2.5 μg/ml, CSE did not exert a statistically significant effect in this assay. For correlation, RT-qPCR was performed to quantify iNOS mRNA levels in IL-1ß-stimulated SW1353 and primary chondrocytes as well as in LPS-stimulated THP-1 cells. As shown in Figure 3C and Table 2, 10 ng/ml IL-1ß markedly stimulated iNOS expression in SW1353 cells and primary chondrocytes, respectively. Pretreatment with CSE dose-dependently suppressed iNOS expression to control levels, supporting the results obtained with the Griess assay. A similar, albeit not significant, trend was also observed in THP-1 cells (Figure 2C).

3.5. Effect of CSE on COX-2 transcription via p65/p50-mediated COX-2 promoter activation

As shown in Figure 3D, IL-1β-induced COX-2 expression was significantly reduced by CSE pretreatment in SW1353 chondrocytes. Accordingly, IL-1ß-induced COX-2 mRNA levels were reduced in a dose-dependent manner by CSE in human primary chondrocytes (Table 2). Previous studies have shown that IL-1β activates COX-2 expression via NF-κB signaling in different cell types, including chondrocytes (Allport et al., 2000; Thomas et al., 2000; Croffort et al., 1997). Therefore, in order to elucidate the mechanism underlying COX-2 suppression by CSE, SW1353 cells were co-transfected with a COX-2 reporter construct and NF-κB p65/p50 expression vectors in the presence or absence of CSE. As shown in Figure 6, the basal COX-2 promoter activity was reduced by 2.5 and 5 μg/ml CSE, although the differences were not statistically significant. Overexpression of the NF-κB subunits p50 and p65 significantly enhanced the COX-2 promoter activity (3.4±0.7 fold, $p<0.05$) whereas the p65/p50-driven COX-2 transactivation was dose-dependently suppressed by CSE (0.5-10 μg/ml), indicating that the anti-inflammatory effects of CSE are, at least in part, derived from its actions on NF-κB signaling.

4. Discussion

It is now accepted that inflammatory processes play a role in the pathophysiology of OA. Indeed, different studies have shown that joint tissue cells, including synovial fibroblasts, synovial macrophages and chondrocytes, produce proinflammatory cytokines, chemokines and other proinflammatory mediators that will in turn result in an inflammatory environment that drives the upregulation of cartilage-degrading MMPs and ADAMTSs (Bodenson et al., 2006; Tsuruda et al., 2004).

Using human OA chondrocytes and THP-1 macrophages, the present study demonstrates for the first time that CSE is a potent inhibitor of proinflammatory mediators in the context of joint inflammation. Several lines of evidence have previously demonstrated the anti-inflammatory potential of *Caesalpinia sappan* extracts and their isolated compounds. In LPS-stimulated RAW264.7 mouse macrophages, Hu and coworkers found that brazilin, the main constituent of CSE, suppressed the release of IL-1ß, TNF-α, NO, and PGE_2 and they suggested that these effects are mediated by Heme oxygenase-1 (Hu et al., 2009). Washiyama *et al*. compared seven compounds isolated from a methanolic extract of Sappan lignum in mouse macrophage-like J774.1 cells and demonstrated inhibitory effects on the expression of inflammatory mediators (Washiyama et al., 2009). More importantly, *in vivo* data from mouse and rat models further support our findings on the anti-inflammatory activity of *Caesalpinia sappan* isolates (Washiyama et al., 2009; Shen et al., 2007). Only recently, a 70% ethanolic extract of *Caesalpinia sappan* has been evaluated in a human cell model of TNF-α-stimulated umbilical vein endothelial cells (Lee et al., 2010). Our present study extends these results to human macrophages and shows that CSE down-regulates IL-1ß and TNF-α mRNA levels in activated THP-1 cells. This result is of interest since synovial macrophages are known to contribute to the pathogenic cascade that leads to OA and rheumatoid arthritis as a major source of inflammatory cytokines and reactive oxygen intermediates (Bondeson et al., 2006). Targeting macrophages using anti-inflammatory phytopharmaceuticals may, therefore, represent a strategy to alleviate synovitis and deleterious mediators in the synovial fluid.

Besides synovial macrophages, OA chondrocytes are a promising target of anti-inflammatory agents. Our present results clearly document that the addition of CSE effectively suppresses the over-expression of IL-1ß and TNF-α in stimulated OA chondrocytes as well as SW1353 cells in vitro, indicating that CSE contains one or more active components that may act as potent anti-inflammatory agents in human chondrocytes. Most likely, brazilin ((6aS,11bR)-7,11b-Dihydro-6H-indeno[2,1-c]chromene-3,6a,9,10-tetrol) contributes to the described anti-inflammatory action of CSE. In the present study, we have isolated brazilin from CSE and identified its structure using ^{1}H NMR. Interestingly, we found that CSE suppressed the IL-1ß-mediated upregulation of TNF-α mRNA levels to a significantly higher extent than NR24. This finding correlates with the lower content of brazilin in NR24 as demonstrated using HPLC analysis and supports the role of brazilin in the anti-inflammatory action of CSE in human chondrocytes.

One of the biological messengers associated with inflammation is NO. NO is biosynthesized endogenously from L-arginine by nitric oxide synthases and can activate transcription factors or protein kinases in a cGMP-dependent manner. Although NO might also regulate physiological processes in chondrocytes, including collagen type X expression and alkaline phosphatase activity, several reports have shown that OA chondrocytes overexpress iNOS and that the resulting excess NO production by OA chondrocytes is a contributing factor to OA cartilage degradative processes (Abramson, 2008; Studer et al., 1999). Experiments in other cell types have indicated that the anti-inflammatory activity of CSE involves the reduction of NO synthesis via inhibition of iNOS mRNA expression (Washiyama et al., 2009; Bae et al., 2005). The latter led us to explore the action of CSE in human OA chondrocytes where, in agreement with the aforementioned reports, we demonstrated that the presence of the extract abrogates IL-1ß-mediated NO production by suppressing iNOS transcription. This suggests that CSE might alleviate deleterious effects associated with over-expression of NO such as the inhibition of collagen and proteoglycan synthesis and the induction of apoptosis (Abramson, 2008).

Furthermore, inflammatory cytokines increase the production of prostaglandins via the induction of COX-2 and microsomal PGE synthase 1 (Abramson and Attur, 2009). Attur

and coworkers reported that PGE_2 decreased proteoglycan synthesis and enhanced matrix degradation in OA cartilage explants (Attur et al., 2008). RT-qPCR results reported in the present study indicate that CSE is able to attenuate IL-1ß-induced COX-2 mRNA expression in SW1353 and primary chondrocytes. This result correlates well with studies on CSE and brazilin in mouse macrophages, demonstrating that the potential anti-inflammatory effects of the agents involve the inhibition of PGE_2 production (Hong et al., 2002; Sasaki et al., 2007). Aiming to further elucidate the mechanisms underlying the anti-inflammatory action of CSE in chondrocytes, we focused on the role of NF-κB in COX-2 promoter activation. The NF-κB signaling is activated by pro-inflammatory stimuli including IL-1ß and TNF-α. Activated NF-κB subunits, such as the RelA (p65)/p50 heterodimer, are translocated to the nucleus, where they bind to target DNA sequences and regulate the expression of a number of genes, including COX-2, iNOS, IL-1β and TNF-α. The COX-2 promoter contains at least four putative NF-κB binding sites and it has been shown that NF-κB modulates COX-2 expression in different cell types, including epithelial cells, rheumatoid synoviocytes and articular chondrocytes (Allport et al., 2000; Thomas et al., 2000; Croffort et al., 1997). In the present study, co-transfection experiments utilizing a COX-2 promoter luciferase construct and p65/p50 expression vectors in the presence or absence of CSE, revealed that CSE strongly inhibited the p65/p50-driven COX-2 transactivation in a dose-dependent manner. This result suggests that CSE interferes with the DNA binding activity of NF-κB subunits, thereby blocking COX-2 transcription. This finding is in agreement with a previous study by Bae et al. showing that brazilin inhibited the DNA binding activity of NF-κB and AP-1 in LPS-stimulated mouse macrophages (Bae et al., 2005).

Finally, it should be noted that the present study is limited in terms of its in vitro experimental design and that extrapolations to in vivo conditions should be carried out with caution. Future studies should aim to define the in vivo actions of CSE using animal models of arthritic diseases, and to further delineate the molecular mechanisms evoked by CSE and its specific constituents. As any other phytochemical drug, CSE or CSE-derived compounds will also require rigorous toxicological and pharmacokinetic testing as well as clinical verification of the findings obtained in vitro.

5. Conclusion

In this study, we demonstrated that CSE possesses anti-inflammatory activity in human macrophages and OA chondrocytes. In these cells, CSE substantially suppresses the expression of inflammatory mediators including IL-1ß and TNF-α at the mRNA level. In addition, we show that the anti-inflammatory actions of CSE involve, at least in part, the inhibition of NO production via iNOS downregulation as well as the inhibition of COX-2 promoter activation by interfering with the canonical NF-κB (p65/p50) signaling pathway. Therefore, we suggest that CSE or CSE-derived compounds should be further developed as therapeutically effective agents or lead structures for future treatment of inflammation in OA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

OA	Osteoarthritis
CSE	Ethanolic *Caesalpinia sappan* extract
IL-1ß	Interleukin-1 beta
LPS	Lipopolysaccharide
NO	Nitric oxide
TNF-α	Tumor necrosis factor- alpha
iNOS	Inducible nitric oxide synthase
COX-2	Cyclooxygenase-2
NF-κB	Nuclear factor-kappa-B
ECM	Extracellular matrix
MMPs	Matrix metalloproteinases
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
PGE_2	Prostaglandin E_2
NSAIDs	Nonsteroidal anti-inflammatory drugs
DMEM	Dulbecco’s modified Eagle’s medium
FCS	Fetal calf serum
PMA	Phorbol 12-myristate 13-acetate
RT-qPCR	Quantitative real-time RT-PCR
ITS	Insulin-Transferrin-Selenium
RIN	RNA integrity numbers
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	Beta actin
HPRT1	Hypoxanthine phosphoribosyl-transferase I
SDHA	Succinate dehydrogenase complex, subunit A
B2M	Beta-2-microglobulin
PC	Primary chondrocytes

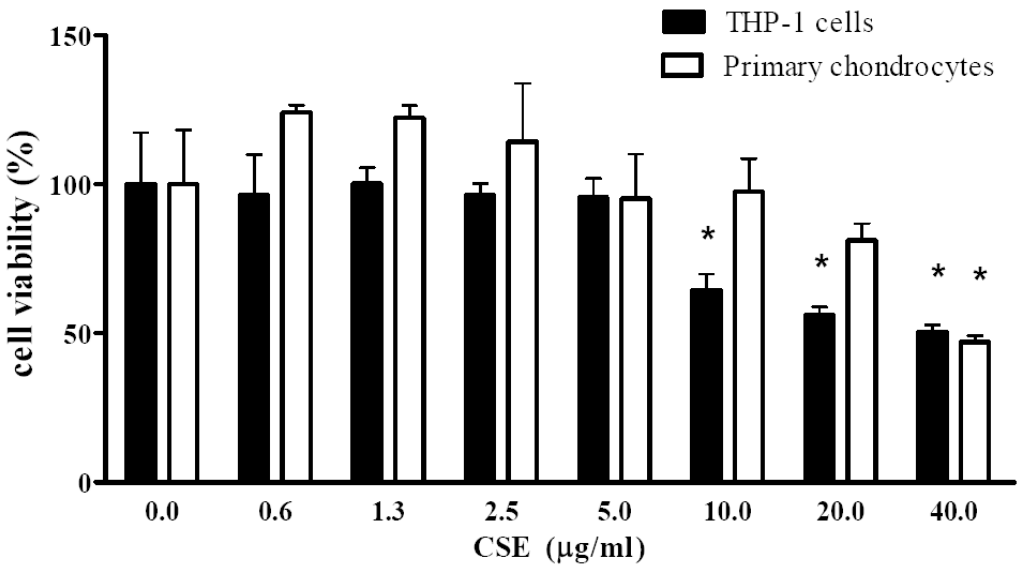

Figure 1. Cytotoxic effect of CSE in differentiated THP-1 cells and primary chondrocytes

Cells were incubated for 72 h with various concentrations of CSE (0.625-40 μg/ml) and the viability was determined using MTT-based assays. Each value represents the mean ± SD. * indicates significant differences as compared to untreated cells ($p < 0.05$).

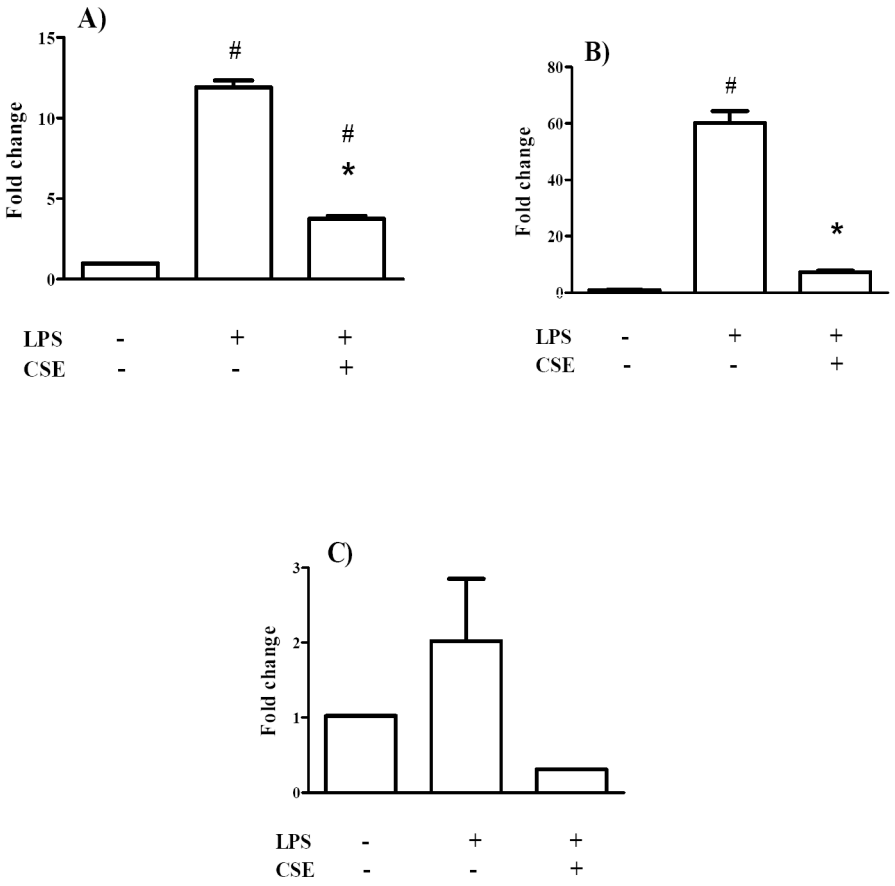

Figure 2. CSE regulates IL-1ß (A), TNF-α (B) and iNOS (C) mRNA in THP-1 cells

THP-1 cells were differentiated to macrophages using PMA and then incubated with 10 ng/ml IL-1ß alone or following pretreatment with 5 μg/ml CSE. The alterations of mRNA levels were analyzed using RT-qPCR. Results are expressed as fold changes (mean ± SD) versus controls. * indicates significant differences compared to cells treated only with IL-1ß ($p < 0.05$). # indicates significant differences compared to untreated cells ($p < 0.05$).

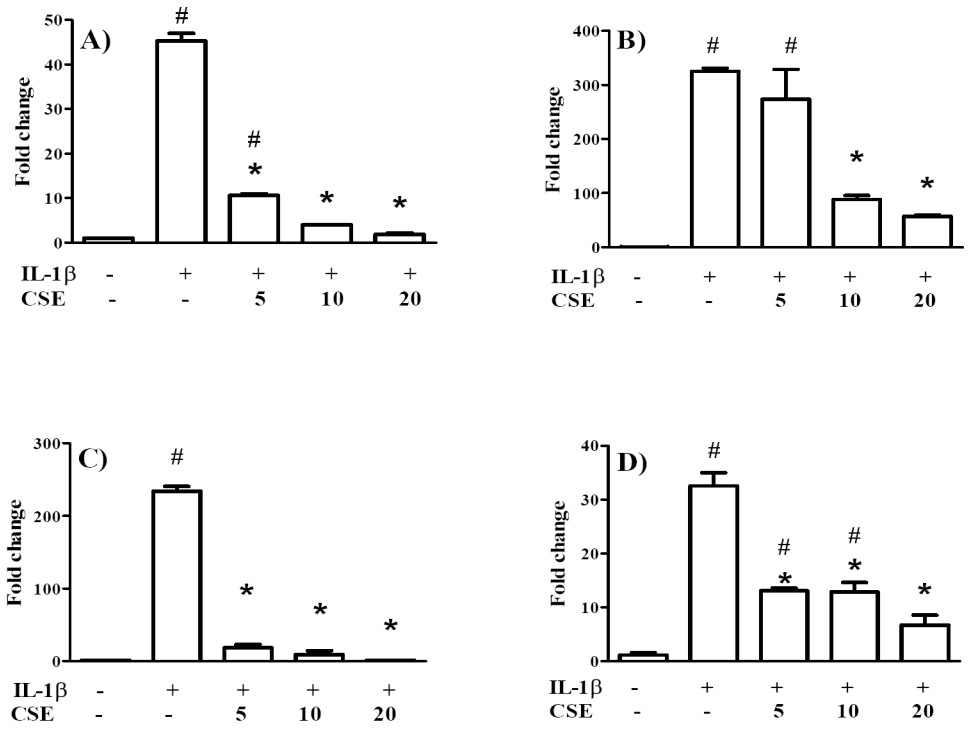

Figure 3. CSE regulates IL-1ß (A), TNF-α (B) iNOS (C) and COX-2 (D) mRNA in SW1353 chondrocytes

Cells were preincubated with 5, 10 or 20 μg/ml CSE for 1 h prior to stimulation with 10 ng/ml IL-1ß for 6 h. The alteration of mRNA levels was analyzed using RT-qPCR. Results are expressed as relative quantities (mean ± SD) compared to controls. * indicates significant differences compared to cells treated only with IL-1ß ($p < 0.05$). # indicates significant differences compared to untreated cells ($p < 0.05$).

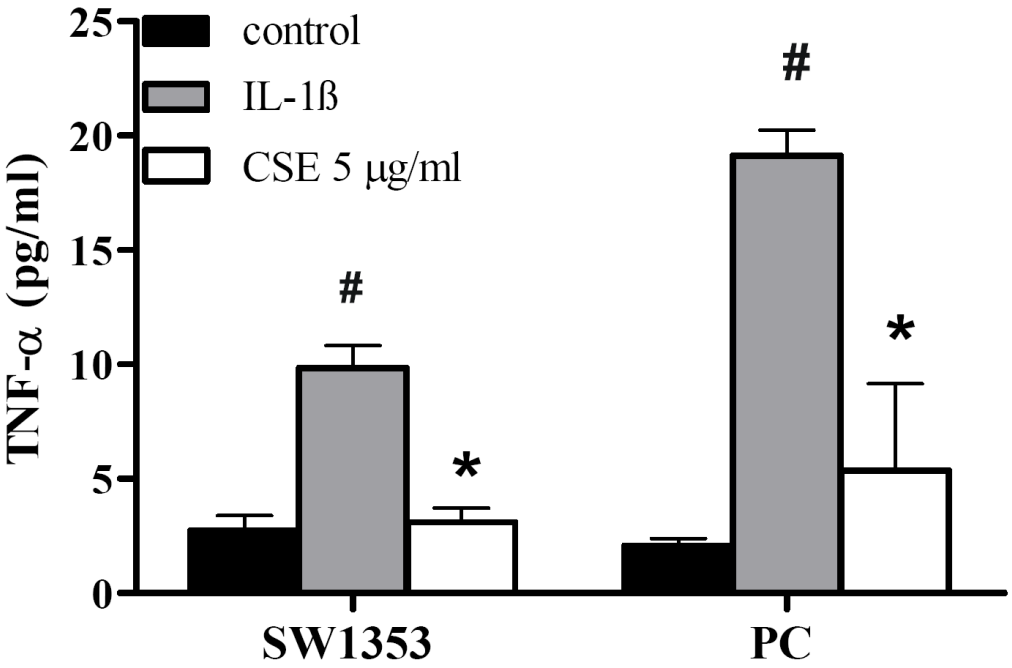

Figure 4. CSE regulates IL-1ß-induced TNF-α production in SW1353 and primary chondrocytes

After 1 h preincubation with 5 μg/ml CSE, cells were treated with 10 ng/ml IL-1ß for additional 48 h. TNF-α levels in the cell culture medium were analyzed using ELISA assay. Primary cells were isolated from 2 donors and analyzed separately. Data are presented as relative quantities (mean ± SD) compared to controls. * indicates significant differences compared to cells treated only with IL-1ß ($p < 0.05$). # indicates significant differences compared to untreated cells ($p < 0.05$).

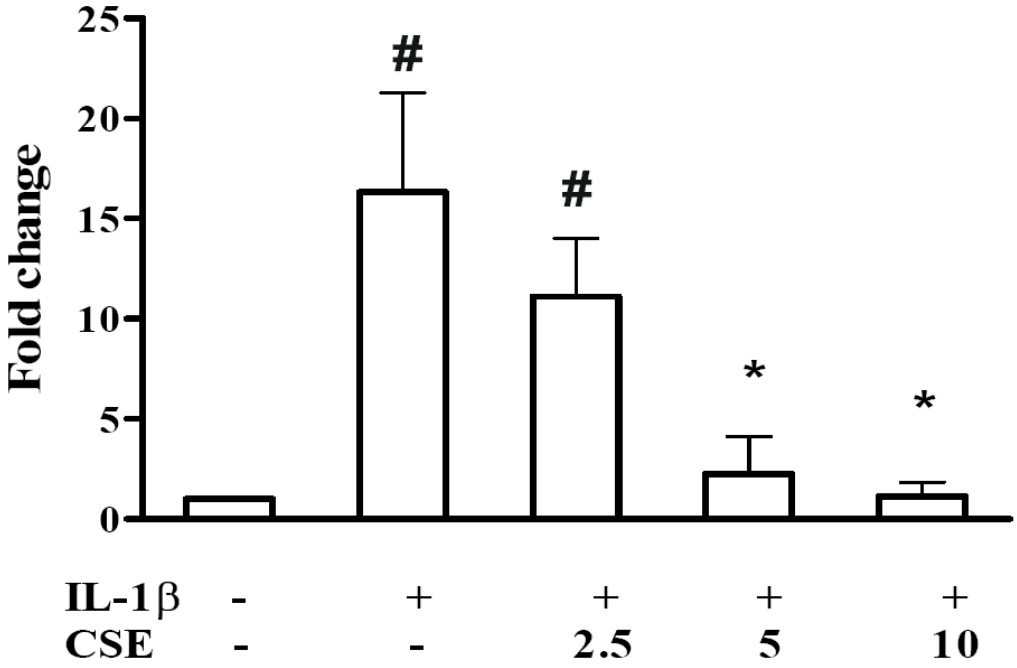

Figure 5. CSE regulates IL-1ß-stimulated NO production in primary chondrocytes

Primary cells were isolated from 3 donors and analyzed separately. Cells were incubated with 2.5, 5 and 10 μg/ml CSE for 1 h prior to coincubation with 10 ng/ml IL-1ß for 24 h. The nitrite concentration in the culture medium was measured using the Griess assay. Data are presented as relative quantities (mean ± SD) compared to controls. * indicates significant differences compared to cells treated only with IL-1ß ($p < 0.05$). # indicates significant differences compared to untreated cells ($p < 0.05$).

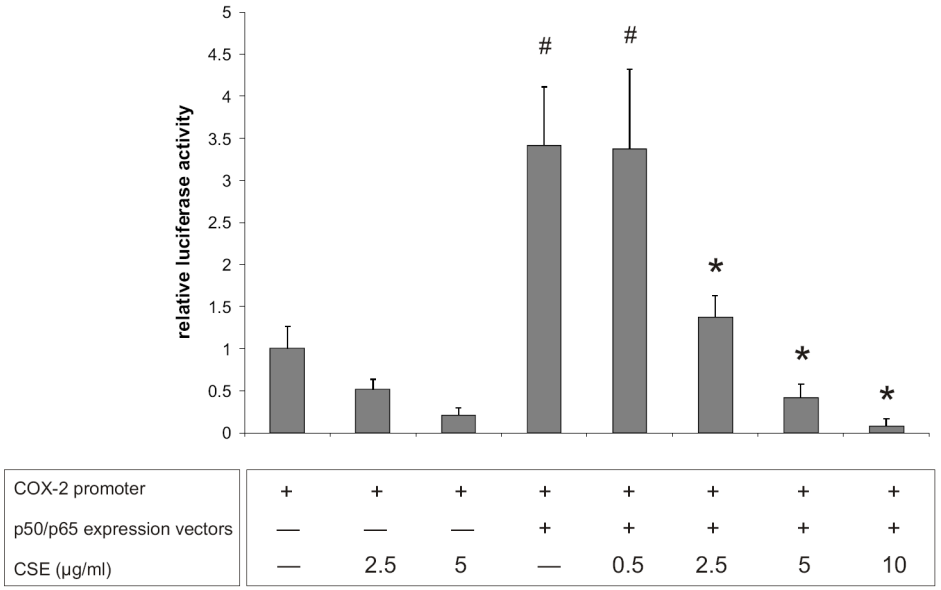

Figure 6. CSE inhibits p65/p50-mediated COX-2 promoter activation in chondrocytes

SW1353 chondrocytes were transfected with the pXP2 luciferase construct containing the -170/+103 COX-2 promoter or cotransfected with NF-κB p50 and p65 expression vectors. Data shown are means of triplicate measurements of luciferase activity from two independent experiments.

# indicates significant differences with respect to cells transfected with the COX-2 reporter construct and the pCI empty vector ($p < 0.05$). * indicates significant differences with respect to cells co-transfected with the COX-2 promoter and the p65/p50 expression vectors ($p < 0.05$).

Table 1

Details on RT-qPCR primers

Gene symbol	Primer sequences	Efficiency	Amplicon size	NM-number
IL-1ß	Fw: CCTATTACAGTGGCAATGAGGATG Rv: AGTGGTGGTCGGAGATTCG	95.1%	132bp	NM_000576
TNF-α	Fw: TCAGCAAGGACAGCAGAGG Rv: CAGTATGTGAGAGGAAGAGAACC	96%	126bp	NM_000594
iNOS	Fw: GTTCTCACGGCACAGGTCTC Rv: GCAGGTCACTTATGTCACTTATC	94.6%	127bp	NM_000625
COX-2	Fw: CCGAGGTGTATGTATGAGTGTG Rv: TGTGTTTGGAGTGGGTTTCAG	96.5%	136bp	NM_000963

Table 2

CSE regulates IL-1ß, TNF-α, iNOS and COX-2 mRNA in primary chondrocytes

Cells were preincubated with 5, 10 or 20 μg/ml CSE for 1 h prior to stimulation with 10 ng/ml IL-1ß for 6 h. The alteration of mRNA levels was analyzed using RT-qPCR. Data from 3 donors (PC1, 2, 3) are presented as relative quantities (mean ± SD) as compared to controls.

		control	IL-1ß	IL-1ß + 5 μg/ml CSE	IL-1ß + 10 μg/ml CSE	IL-1ß + 20 μg/ml CSE
IL-1ß	PC1	1.00 ± 0.04	218.94 ± 6.95#	2.66 ± 0.01*	0.43 ± 0.06*	0.52 ± 0.11*
	PC2	1.00 ± 0.03	8359.31 ± 113.81#	752.09± 68.18 *	15.29 ± 1.73*	No Cq
	PC3	1.00 ± 0.01	910.01 ± 8.26#	229.19 ± 41.38	255.77 ±2.32*	12.93 ± 1.06*
TNF-α	PC1	1.00 ± 0.78	468.07 ± 27.37#	4.82 ± 0.63*	3.68 ± 0.69*	7.64 ± 0.86*
	PC2	1.00 ± 0.03	1013.48 ± 136.44#	128.52 ± 4.63*	22.13 ± 0.30*	37.0 ± 5.16*
	PC3	1.00 ± 0.02	1786.13 ± 200.57#	501.60 ± 51.84*#	154.04± 16.61*	28.88 ± 4.92*
iNOS	PC1	1.00 ± 0.04	659.17 ± 28.86#	48.86 ± 4.04*	1.22 ± 0.01*	0.19 ± 0.02*
	PC2	1.00 ± 0.10	20014.19 ± 4442.74#	3297.77 ± 96.27*#	163.84 ± 18.30*	0.88 ± 0.05*
	PC3	1.00 ± 0.11	7363.67 ± 644.13#	2952.28 ± 14.37#	4047.04 ± 666.50*#	123.64 ± 9.61*
COX-2	PC1	1.00 ± 0.01	96.10 ± 2.23#	21.39 ± 2.28*#	9.61 ± 0.53*#	2.93 ± 0.40*
	PC2	1.00 ± 0.04	121.84± 36.81#	71.07 ± 7.26#	23.19 ± 0.32*	9.07 ± 1.26*
	PC3	1.00 ± 0.13	304.77 ± 11.33#	134.24 ± 9.35*#	128.23 ± 3.58*#	28.36 ± 0.92*#

* indicates significant differences compared to cells treated only with IL-1ß ($p < 0.05$).

# indicates significant differences compared to untreated cells ($p < 0.05$)

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