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Inhibition of cyclooxygenase-2 expression and prostaglandin E_2 production in chondrocytes by avocado soybean unsaponifiables and epigallocatechin gallate

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Summary

Objective: To evaluate the anti-inflammatory effect of the combination of avocado soybean unsaponifiables (ASU) and epigallocatechin gallate (EGCG) on cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) production in cytokine-activated equine chondrocytes.

Methods: Production of type II collagen and aggrecan was verified by immunohistochemistry and Western blot. Chondrocytes were incubated with: (1) control media alone, (2) ASU (4 μg/ml; 8.3 μg/ml), (3) EGCG (4, 40, 400 ng/ml), or (4) the combination of ASU and EGCG for 24 h. Cells were next incubated with control medium alone or with IL-1β (10 ng/ml) and TNF-α (1 ng/ml). COX-2 gene expression by real-time PCR analysis and NF-κB nuclear translocation by immunohistochemistry were performed after 1 h of incubation. PGE_2 production was determined by immunoassay after 24 h of incubation.

Results: Equine chondrocytes responded to cytokine activation by up-regulated gene expression of COX-2 and increased PGE_2 production. Activation was associated with NF-κB translocation. Individually, ASU and EGCG marginally inhibited COX-2 expression and PGE_2 production in activated chondrocytes. In contrast, the combination of ASU and EGCG reduced COX-2 expression close to non-activated control levels and significantly inhibited PGE_2 production. These reductions were statistically greater than those of ASU or EGCG alone. The inhibition of COX-2 expression and PGE_2 production was associated with inhibition of NF-κB translocation.

Conclusion: The present study demonstrates that the anti-inflammatory activity of ASU and EGCG is potentiated when used in combination. This combination may offer an attractive supplement or alternative to non-steroidal anti-inflammatory drugs (NSAIDs) in the management of osteoarthritis.

Key words: Chondrocytes, COX-2, PGE_2, ASU, EGCG, NF-κB.

Introduction

Osteoarthritis (OA) is the most common cause of morbidity in horses and accounts for an estimated 60% of lameness problems[1,2]. OA compromises performance and ultimately leads to retirement of many equine athletes[3]. This degenerative condition is characterized by erosion of articular cartilage, inflammation of the synovial membrane, and resorption of the underlying subchondral bone[4–6]. The pathological changes in OA are associated with an excess production of pro-inflammatory mediators which shift the balance between the synthesis and degradation of matrix components towards progressive destruction of joint tissue[7–9].

A key player in the pathogenesis of OA is prostaglandin E_2 (PGE_2)[10]. This molecule contributes to several distinct pathological features of OA including joint inflammation, tissue destruction, and inflammatory pain. PGE_2 also plays a regulatory role and can induce the production of other pro-inflammatory mediators including cytokines, nitric oxide, and connective tissue degrading enzymes. Due to its ability to upregulate metalloproteinases (MMPs), PGE_2 contributes to the breakdown of cartilage extracellular matrix[9,11]. In addition, PGE_2 promotes bone resorption and osteophyte formation[11,12]. PGE_2 sensitizes nociceptors on peripheral nerve endings thereby contributing to the development of inflammatory pain[13].

PGE_2 levels are locally regulated by the inducible cyclooxygenase-2 (COX-2) enzyme, a nitric oxide synthase in chondrocytes that inhibit cartilage and proteoglycan degradation possibly through inhibition of degradative MMPs[14–18]. In pathologic conditions such as OA, COX-2 expression is up-regulated with a concomitant increase in PGE_2 production. The central role of COX-2 and PGE_2 in the pathophysiology of OA is reflected in the widespread use of selective COX-2 inhibitors and a variety of non-selective non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of the disorder. However, prolonged administration of these drugs has adverse side effects including gastrointestinal

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pathologies and disruption of cartilage proteoglycan metabolism[19,20]. Studies in human and animal models have demonstrated impaired bone healing and repair with the use of COX inhibitors[21,22]. Therefore, there is a need for alternative treatments for the management of OA that do not center on the use of NSAIDs to inhibit the production of PGE_2 and other pro-inflammatory mediators.

In addition to pro-inflammatory mediators such as cytokines and prostaglandins, reactive oxygen species (ROS) have also been implicated in the pathogenesis of OA[23–25]. Oxidative stress, induced by ROS including nitric oxide and hydrogen peroxide, have been shown to induce chondrocyte apoptosis and cartilage degeneration. Moreover, ROS have been reported to activate signal transduction pathways that lead to an increased production of pro-inflammatory mediators including cytokines and prostaglandins[23,26–29]. Studies *in vitro* have demonstrated a linkage between the pathways involved in the production of ROS and pro-inflammatory mediators[30,31]. These studies support the notion that agents capable of inhibiting both oxidative stress and inflammation pathways would be particularly useful in the management of OA.

Many studies have documented the benefits of the nutritional supplement avocado soybean unsaponifiables (ASU) for promoting joint health and the management of OA. ASU is derived from avocados and soybeans and has been used for years to manage joint pain[32]. ASU is a fraction of avocado and soybean oils which does not produce soap after hydrolysis[32]. Clinical studies have reported beneficial effects of ASU in human and equine OA patients as well as in experimental animal models of OA[1,32–35]. The mechanisms that could account for the beneficial effects of ASU for OA have been studied *in vitro* using bovine and human joint tissue cells. These studies showed that ASU inhibits the expression and production of cytokines, chemokines, PGE_2, nitric oxide, and MMPs[36–40]. ASU also exerts anabolic effects on cartilage metabolism by enhancing synthesis of cartilage matrix components while suppressing their degradation[37].

Evidence indicating that joint diseases are associated with increased production of ROS suggests that agents with anti-oxidant activity may have beneficial effects in the treatment of OA. Epigallocatechin gallate (EGCG), a major anti-oxidant component of green tea, has been reported to inhibit the onset and severity of collagen induced arthritis in mice[41,42]. *In vitro* studies have demonstrated both anti-inflammatory and anti-oxidant activities of EGCG, and that EGCG suppresses COX-2. Based on these studies, we hypothesize that the combination of ASU with EGCG may have a broader spectrum of anti-inflammatory activities than either preparation alone. In particular, we sought to determine whether EGCG may enhance the anti-inflammatory effects of ASU by virtue of its suppressive action of COX-2 induction. We utilized the equine chondrocyte culture model to determine the inhibition of COX-2 expression and PGE_2 production. The benefit of combining the two agents together may offer an alternative for the management of OA.

Materials and methods

CELL CULTURE

Articular cartilage was harvested and aseptically diced into <5 mm pieces from three adult horses. The cartilage was then digested in type II collagenase (110 U/ml, Gibco Invitrogen, Carlsbad, CA, USA) for 12–18 h at 37°C, 5% CO_2. Chondrocytes were filtered through a wire mesh screen to remove debris and rinsed four times with Hank's Balanced Salt Solution (American Type Culture Collection (ATCC) Manassas, VA, USA). Cells were counted and assessed for viability using the Trypan-blue exclusion method. Chondrocytes were propagated in monolayer culture until confluency in media composed of Dulbeccos' Modified Eagle's basal medium (Sigma; St. Louis, MO, USA) supplemented with 10% v/v fetal bovine serum (Gemini Bio-Products; Woodland, CA, USA), 300 μg/ml L-glutamine (Sigma), 30 μg/ml antibiotic/antimycotic (Sigma), and 3.7 g/L sodium bicarbonate (Sigma). The final pH of the media was adjusted to 7.4.

PHENOTYPE ANALYSIS BY IMMUNOHISTOCHEMISTRY AND WESTERN BLOT ANALYSIS

Chondrocytes were plated on microscope slides and fixed with 10% v/v paraformaldehdye. Slides were then incubated with goat anti-type I collagen, anti-type II collagen, or anti-aggrecan antibodies (Southern Biotechnology Associates; Birmingham, AL USA). The slides were next washed in phosphate buffered saline (PBS, Gibco Invitrogen, Carlsbad, CA, USA) three times and incubated with fluorescein isothiocyanate (FITC) labeled anti-goat antibodies. Immunostaining was visualized using a Nikon Eclipse epifluorescent microscope TE200. To identify secreted collagen and aggrecan, spent culture media were electrophoresed on 4–15% (w/w) sodium dodecyl sulfate-polyacrylamide gels. Following electrophoresis, the gels were electrophoretically transferred to PolyVinylidine DiFluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) in Tris-glycine buffer, pH 8.5, containing 20% v/v methanol. Blotted PVDF membranes were washed twice with deionized water and stained using a chromogenic Western blot immunodetection kit (Gibco Invitrogen). To block non-specific staining, membranes were treated with a blocking solution provided with the immunodetection kit following the instructions of the manufacturer. PVDF membranes were then processed for immunostaining using goat anti-collagen type II, type I antibodies, or anti-aggrecan (Southern Biotechnology Associates) in combination with an alkaline phosphatase labeled rabbit anti-goat antibody with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NTB) (Gibco Invitrogen) as the substrate.

EXPERIMENTAL DESIGN

ASU (ASU®-NMX 1000; Nutramax Laboratories, Inc., Edgewood, MD, USA) was dissolved in 100% ethanol (Sigma) and diluted in media to achieve the required final concentration. The control media containing the same ethanol concentration did not cause toxicity or inflammation. The composition of ASU was previously described and concentration of ASU (8.3 μg/ml) used in this study was previously shown to exert significant anti-inflammatory effect[36,37]. We also evaluated ASU at a concentration of 4 μg/ml. The concentrations of EGCG used were based on reported detectable levels in the blood following intake of green tea EGCG and on reported *in vitro* studies[18,43,44]. EGCG was commercially obtained (Indena). The range of concentrations in the present *in vitro* study appear to be achievable *in vivo* based on the published pharmacokinetic studies[43,44].

Chondrocytes (5×10^5) were seeded onto 6-well plates for 24 h and were incubated with: (1) control media alone, (2) ASU (4 or 8.3 μg/ml), (3) EGCG (4–400 ng/ml), or (4) the combination of the two agents for another 24 h. Following this treatment, cultures were incubated with control media alone, or activated with cytokines interleukin-1-beta (IL-1β) (10 ng/ml) and tumor necrosis factor-alpha (TNF-α) (1 ng/ml) for 1 h to measure COX-2 by real-time polymerase chain reaction (RT-PCR), or for 24 h to measure secreted PGE_2 levels by immunoassay.

TOTAL RNA ISOLATION

Total cellular ribonucleic acid (RNA) was isolated by lysing the cells with TRIzol® reagent (Gibco Invitrogen) and extracted with chloroform (Sigma). Following vigorous agitation and a 3 min incubation at room temperature, samples were centrifuged and the aqueous phase containing RNA was collected. The RNA was precipitated with isopropyl alcohol (Sigma) and resuspended in RNase-free water (Gibco Invitrogen). Total RNA was quantified with UV spectrophotometry (Molecular Devices, Sunnyvale, CA, USA) and evaluated for RNA concentration and integrity.

cDNA SYNTHESIS

For each sample, 1 μg of total RNA was converted to complementary DNA (cDNA) using Moloney-Murine Leukemia Virus reverse transcriptase from the Advantage RT-for-PCR kit (BD Biosciences Clontech, Mountain View, CA, USA). RT was carried out at 42°C for 60 min followed by heating at 94°C for 5 min to stop the cDNA synthesis reaction and to destroy any DNase activity.

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

RT-PCR was carried out by combining 2 μl of cDNA and reagents from the iQ™ SYBR Green Supermix Kit (Bio-Rad, Hercules, CA) to give a total volume of 25 μl. The primer sequences used were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (forward, GTTTGTGATGGGCGTGAACC;

reverse, TTGGCAGCACCAGTAGAAGC) a house-keeping gene, and COX-2 (forward, ATACCAAAACCGCATTGCCG; reverse, TCTAACTCCGCAGCCATTTC). Reaction conditions used for both primers are as follows: 95°C for 3 min, then 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s repeated for 40 cycles, and then 95°C for 1 min, 55°C for 1 min, and 55°C for 30 s repeated for 81 cycles to obtain a melt curve. Thermal cycling was performed using an iQ5 Multicolor RT-PCR Detection System (Bio-Rad). Results were normalized to the GAPDH house-keeping gene. Four independent cellular experiments were performed and the average of these experiments are shown in the figures. Each assay was done using triplicate samples.

PGE_2 HIGH SENSITIVITY IMMUNOASSAY

A commercial PGE_2 immunoassay (R&D Systems, Minneapolis, MN USA) was used to quantify secreted PGE_2 levels in the cellular supernatant, according to the manufacturer's instructions. A PGE_2 standard was run in parallel to the supernatant samples. Each assay was performed using triplicate samples. Optical density was measured immediately using the SpectraMAX 340 microplate reader (Molecular Devices; Sunnyvale, CA, USA) at 450 nm with wavelength correction set at 540 nm.

INTRACELLULAR LOCALIZATION OF NF-κB BY IMMUNOFLUORESCENCE

The effect of IL-1β and TNF-α on the nuclear translocation of nuclear factor kappa B (NF-κB) was monitored by immunohistochemistry using an antibody to the p65 subunit of NF-κB (Santa Cruz Biotechnology; Santa Cruz, CA USA). Equine chondrocytes were seeded into 8-chambered cover glass culture plates (Nalge Nunc International) at a density of 1×10^4/well for 24 h. Chondrocytes in each compartment were subjected to various treatments as detailed below and then processed for analysis by fluorescence microscopy *in situ*. To determine the effect of treatments on the nuclear translocation of p65 NF-κB in response to IL-1β and TNF-α exposure, cells were pre-treated with: (1) control media alone or (2) the combination of ASU (8.3 μg/ml) and EGCG (40 ng/ml). Chambers were incubated for 24 h followed by the addition of cytokines for 1 h. Cells were then fixed with 10% v/v formalin for 15 min and washed three times with phosphate buffered saline (Gibco Invitrogen). For immunohistochemical staining, cells were incubated with a 1:100 dilution of rabbit anti-NF-κB (Santa Cruz Biotechnology) in PBS containing 0.1% Triton X-100 (Sigma) overnight at 4°C. After four washes with PBS, cells were incubated with a 1:100 dilution of donkey anti-rabbit IgG labeled with Alexa Fluor-488 (Invitrogen) for 2 h at room temperature. After four washes with PBS, cells in chamber slides were viewed on an inverted fluorescence microscope (Nikon Eclipse TE200) equipped with a digital camera (Nikon Spot Camera). Digital images of five frames were captured for each experimental condition and were imported into Adobe Photoshop. To quantify the number of cells with p65 positive nuclei, digital images were analyzed using the NIH ImageJ software program. Cells with p65 positive and negative nuclei were determined using the cell count function of the program.

STATISTICAL ANALYSIS

Data is presented as the mean ± 1 SD. Pair-wise multiple comparisons was carried out using one-way analysis of variance (ANOVA), Tukey post-hoc using SigmaStat statistical software (Windows Version 3.11)[12] where $P < 0.05$ was considered statistically significant. The SigmaStat program verified that our data is compatible with the assumptions of Normality – Gaussian distribution and homogenous variance. The Figure legends indicate the number of (3–4) runs performed.

Results

PHENOTYPE CHARACTERIZATION OF CHONDROCYTE MONOLAYER CULTURE

Equine chondrocytes proliferated with ease in monolayer culture with 100% viability. The doubling time for monolayer cultures was 3–5 days. Chondrocytes propagated on monolayer cultures at passage three showed elongated, spindle-shaped morphology [Fig. 1(A)]. Immunohistochemical analysis confirmed that chondrocyte cultures continued to produce the ECM components aggrecan and type II collagen [Fig. 1(B) and (C), respectively]. Production of type II collagen was further verified by Western blot [Fig. 1(D)]. Chondrocyte cultures showed negligible production of type I collagen. The high molecular weight aggrecan protein did not enter the gel and could not be visualized on Western blot.

THE EFFECT OF ASU AND EGCG ON COX-2 GENE EXPRESSION IN EQUINE CHONDROCYTES

Chondrocytes responded to cytokine activation with a greater than two-fold increase in COX-2 expression (Fig. 2). Pre-treatment of chondrocyte cultures with ASU (8.3 μg/ml) or EGCG (40 ng/ml) alone did not reduce COX-2 expression compared to activated control levels (Fig. 2). In contrast, the combination of ASU and EGCG

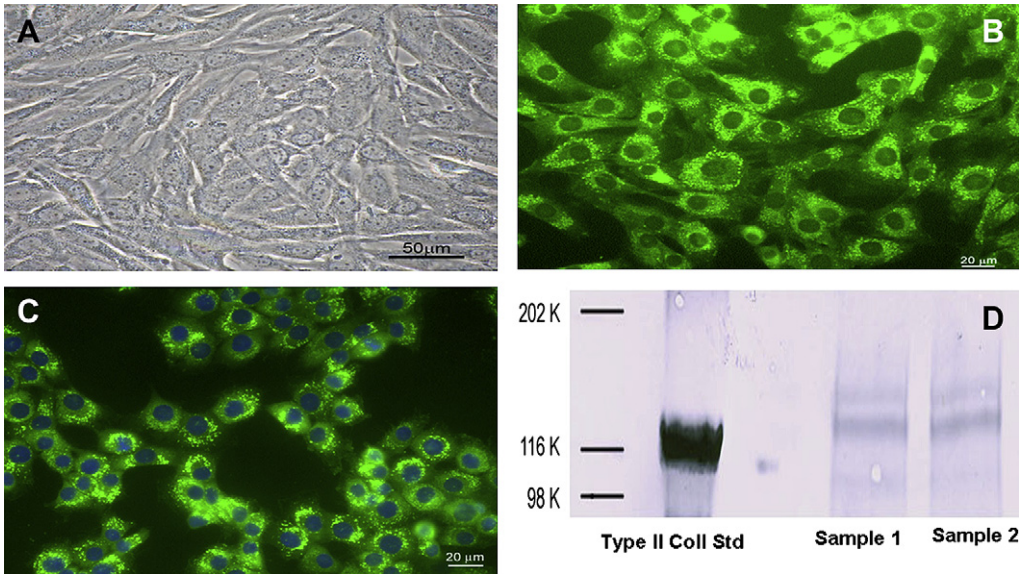

Fig. 1. (A) Phase-contrast photomicrograph of equine chondrocyte culture, (B) Immunostaining for aggrecan, (C) Immunostaining for type II collagen, (D) Western blot for type II collagen.

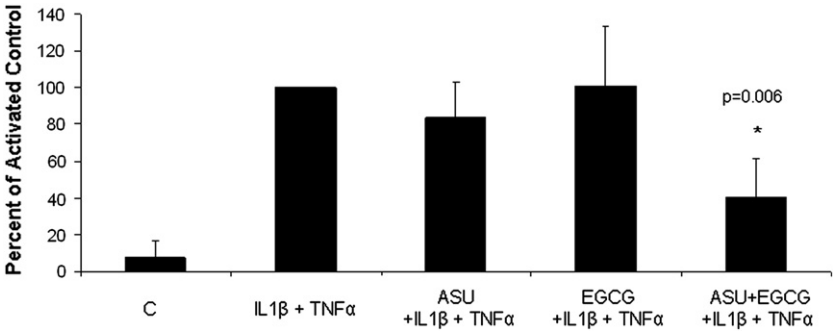

Fig. 2. Effect of ASU (8.3 μg/ml), and EGCG (40 ng/ml) on COX-2 gene expression in cytokine-activated chondrocyte cultures. Chondrocytes were pre-treated with ASU (8.3 μg/ml) and EGCG (40 ng/ml) for 24 h and activated with IL-1β and TNF-α for 1 h. COX-2 gene expression analyzed by RT-PCR according to Methods and was normalized to the activated control. Significance was analyzed using Tukey post-hoc test (mean ± 1 SD, $n = 4$).

($P = 0.006$) reduced COX-2 expression to levels similar to non-activated controls (Fig. 2).

THE EFFECT OF ASU AND EGCG ON PGE_2 PRODUCTION BY EQUINE CHONDROCYTES

Chondrocytes responded to cytokine activation with a significant increase in PGE_2 production ($P < 0.001$, Fig. 3). Pre-treatment with ASU (8.3 μg/ml) or EGCG at concentrations ranging from 4 to 400 ng/ml did not decrease PGE_2 production ($P > 0.05$, Fig. 3). The combination of ASU at 8.3 μg/ml with EGCG at concentrations 4–400 ng/ml significantly reduced PGE_2 production relative to the activated control IL-1β and TNF-α ($P < 0.05$, Fig. 4). Pre-treatment of chondrocytes with 4 μg/ml of ASU did not reduce PGE_2 levels, nor did EGCG at 40 ng/ml. The combination of ASU at 4 μg/ml and EGCG at 40 ng/ml significantly decreased PGE_2 production ($P < 0.001$, Fig. 4).

THE EFFECT OF ASU AND EGCG ON NF-κB TRANSLOCATION

Non-stimulated chondrocyte controls showed strong NF-κB immunostaining throughout the cytoplasm while the nuclei of chondrocytes were unstained as indicated by arrows (Fig. 5, top panel). Following cytokine stimulation, cytoplasmic immunostaining for NF-κB appeared unchanged while the nuclei were intensely stained as indicated by arrows in Fig. 5 (middle panel). This nuclear translocation of NF-κB was identified by intense immunostaining at 1 h. The translocation of NF-κB from cytoplasm to the nucleus visualized by immunostaining was inhibited by pre-treatment of chondrocytes with the combination of ASU and EGCG (Fig. 5 lower panel). Pre-treatment of chondrocytes with the ASU and EGCG combination significantly reduced NF-κB translocation [Fig. 5(B), $P < 0.001$].

Discussion

The main finding of the present paper is that in cytokine-activated equine chondrocyte cultures, the combination of ASU and EGCG inhibits COX-2 expression and PGE_2 production to a greater extent than either compound alone. COX-2 catalyzes the biosynthesis of PGE_2 and has been shown to be overexpressed in joints affected by OA. The increased expression of COX-2 and the concomitant increased production of PGE_2 are considered a major cause of the pathological changes seen in the disease. Accordingly, COX-2 has become an important target for pharmacological interventions aimed at treating OA. In this study, we used equine chondrocyte cultures to identify the effects of two products on the cytokine-stimulated induction of COX-2 and PGE_2 synthesis by these cells. The production of cartilage markers type II and aggrecan was verified in our chondrocyte culture model [Fig. 1(A–D)]. The response of the chondrocyte model to stimulation with IL-1β and TNF-α with significantly increased COX-2 expression and PGE_2 production confirms the continued functional and metabolic activity of these cells.

Previous studies have shown that ASU, as well as EGCG, inhibit cytokine induction of COX-2 expression and PGE_2 production[18,36,37,43,44]. We evaluated several concentrations of ASU and EGCG and found that either preparation alone produced only a marginal inhibitory anti-inflammatory effect in our chondrocyte cultures (Fig. 2–4). The marginal inhibitory effect of ASU or EGCG on COX-2 and PGE_2 production could be due to the distinct origin or purity of these preparations. The discrepancy in effects could also be due to the differences in the cell models and culture conditions used[36–40]. In contrast, the combination of ASU and EGCG at concentrations at which neither one alone inhibits cytokine-stimulated COX-2 and PGE_2, resulted in significant inhibition of COX-2 expression at the transcript level and PGE_2 synthesis (Figs. 2–4). These results demonstrate the dramatic effects of the ASU and EGCG combination in inhibiting the induction of inflammatory markers. That the combination of ASU and EGCG significantly reduced, but did not completely block, COX-2 gene expression and PGE_2 production, further confirms their regulatory role. Their ability to spare some expression of COX-2 and PGE_2 production may avoid the adverse effects of selective COX-2 inhibitors[21,22,45].

Our unexpected observation that ASU and EGCG potentiate their inhibitory effects on cytokine-inducted COX-2 expression and PGE_2 synthesis may be the result of overlapping activities of the two preparations in COX-2 gene regulation. The COX-2 gene contains several regulatory elements for control at the transcriptional level is also regulated at the post-transcriptional level. Binding sites for regulatory transcription factors include NF-κB motifs, AP-1 sites and cyclic adenosine monophosphate (cAMP) response elements[46]. Activation of the NF-κB pathway by cytokines has previously been reported for rabbit and human chondrocytes[47,48]. There are two NF-κB binding sites

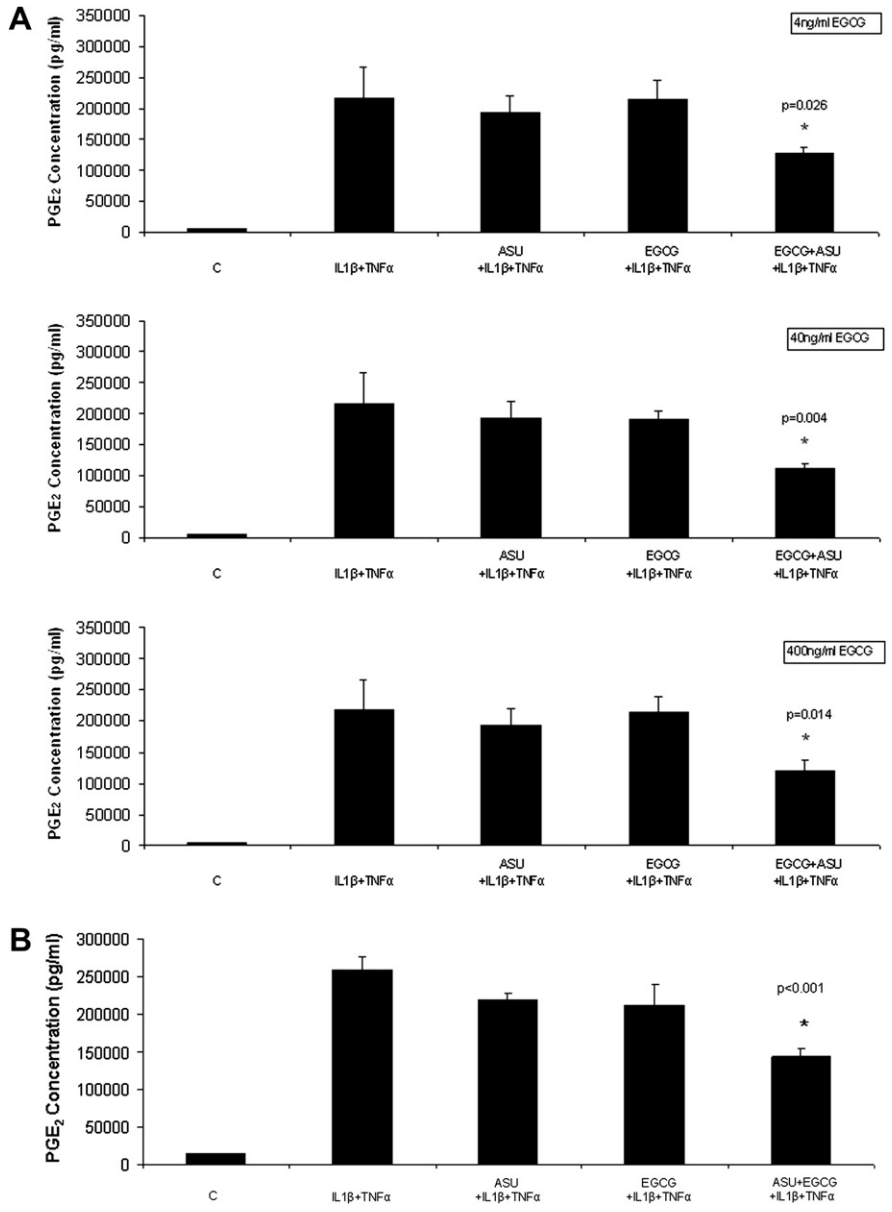

Fig. 3. (A). Effect of ASU (8.3 μg/ml) and EGCG (4, 40, 400 ng/ml) on PGE_2 production in cytokine-activated chondrocyte cultures. Chondrocytes were pre-treated with ASU (8.3 μg/ml) and EGCG (4–400 ng/ml) for 24 h and activated with IL-1β and TNF-α. After an additional 24 h supernatant was collected and assayed for PGE_2 levels. Statistical significance between the activated control and the pre-treated group were analyzed using Tukey post-hoc analysis (mean ± 1 SD, $n = 3$). After an additional 24 h supernatant was collected and assayed for PGE_2 levels. Statistical significance between the activated control and the pre-treated group were analyzed using Tukey post-hoc analysis (mean ± 1 SD, $n = 3$). (B). Effect of ASU (4 μg/ml) and EGCG (40 ng/ml) on PGE_2 production in cytokine-activated chondrocyte cultures. Chondrocytes were pre-treated with ASU and EGCG for 24 h. PGE_2 production was measured from cellular supernatant by chondrocytes activated with IL-1β and TNF-α for 24 h.

on the 5′ flanking region of the COX-2 gene and NF-κB is known to be an essential transcription factor for the induction of COX-2 induction[46]. Inhibition of the NF-κB pathway has previously been shown to attenuate COX-2 expression[49]. Our finding that the cytokine-induced COX-2 expression and PGE_2 in equine chondrocytes was paralleled by translocation of NF-κB from the cytoplasm to the nucleus (Fig. 5) is in line with observations in other species. Previous reports have shown that both IL-1β and TNF-α contribute to the nuclear translocation of NF-κB and have also demonstrated that this translocation can be inhibited by ASU and EGCG. NF-κB has been shown to play a role in stimulating COX-2 expression in synovial tissue[50]. Inhibiting the translocation of NF-κB has long been considered an attractive target for pharmacological or nutraceutical agents to treat chronic inflammatory conditions. Our results demonstrate that nearly complete inhibition of NF-κB translocation can be achieved by the combination of ASU and EGCG.

In view of the multiplicity of promoter elements, it is possible that ASU and EGCG may exert their effects at different regulatory sites of the COX-2 gene[51]. In chondrocytes and

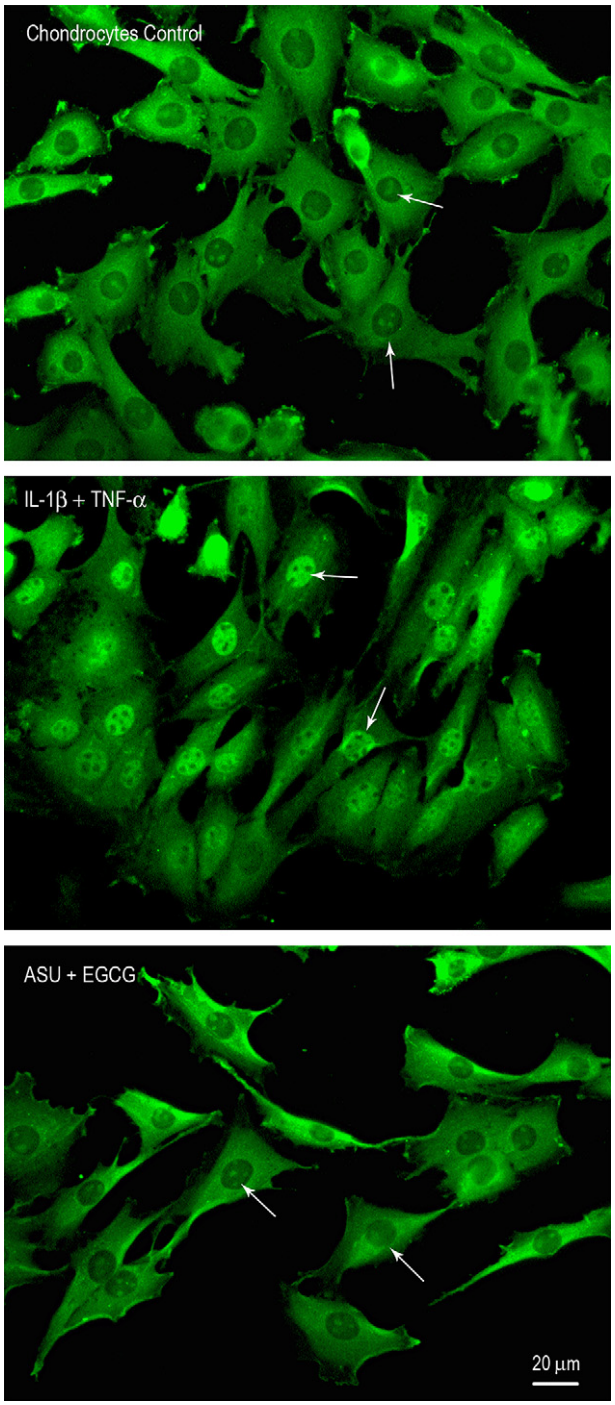

Fig. 4. Immunostaining of chondrocyte cultures for NF-κB translocation. Note immunostaining of cytoplasm in control chondrocytes (top panel). In contrast, cytokine activation induced translocation of immunostaining in the nucleus indicated by arrows (middle panel). Pre-treatment with the combination of ASU (8.3 μg/ml) and EGCG (40 ng/ml) inhibited translocation indicated by more predominant cytoplasmic immunostaining for NF-κB (lower panel). Arrows point to nuclei. Bar = 20 μm.

synovial cells, the mitogen-activated protein kinase (MAPK) pathway is particularly well characterized. An alternative explanation for the potentiated effects of the ASU and EGCG combination observed in this study may be due to

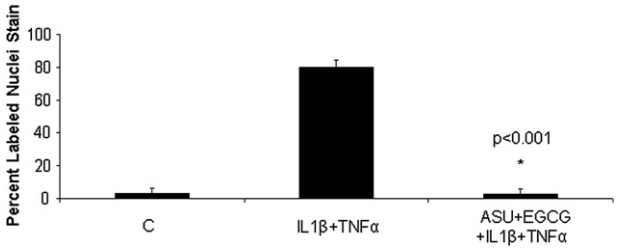

Fig. 5. Percentage of NF-κB nuclear immunostained chondrocytes. pre-treatment with the combination of ASU (8.3 μg/ml) and EGCG (40 ng/ml) inhibited translocation.

a differential effect of the two compounds in the post-translational regulation of COX-2 expression. Inhibitors of mitogen-activated protein kinases have been shown to down-regulate COX-2 expression in human chondrocytes through a mechanism that involves interference with messenger RNA (mRNA) stability[46,52,53]. MAPKs are a family of kinases that are part of the signal transduction pathways which connect inflammatory signals to intracellular responses such as gene expression. In particular, the p38 MAPKs have received a great deal of attention as therapeutic targets for inflammatory diseases[54–56]. Inhibitors of MAPKs suppress inflammatory mediator production and have shown efficacy in experimentally induced arthritis and joint pain.

Preventing COX-2 overexpression is a compelling rationale in the treatment of OA. Our finding that the combination of ASU and EGCG provides a high degree of inhibition of cytokine-induced COX-2 expression and PGE_2 synthesis in cultures of equine chondrocytes suggests that this combination may be effective in the management of OA in horses as well as in other species. The finding that excessive PGE_2 production can be prevented through the action of the combination ASU and EGCG, suggests that the pathophysiological consequences of COX-2 overexpression may be achieved with or without limited use of NSAIDs, in equine OA. The strategy of combining two or more agents may prove to be a promising strategy in cases in which multiple signaling pathways converge onto a single target biological target such as the expression of COX-2[57–60]. Other signaling pathways such as those involved in matrix synthesis and breakdown may also be affected by ASU and EGCG[1,5,9,10]. It would be interesting to determine in future studies whether the combination of the two would have a more profound effect in the regulation of matrix synthesis and degradation following induction of inflammation. Our observation that pre-treatment of chondrocytes with the combination of ASU and EGCG prior to cytokine stimulation inhibiting the inflammatory response suggests two potential benefits. The combination of the two compounds may help modulate the pathogenesis of OA. The robust response of cytokine-activated equine chondrocytes to the combination of ASU and EGCG suggests that such combinations may also offer an intriguing alternative-complementary treatment option for the management of OA.

Conflict of interest

The authors listed below are funded by Nutramax Laboratories Inc but do not hold stocks, or royalties. Carmelita G. Frondoza, Lowella F. Heinecke, Mark W. Grzanna, Angela Y. Au, Catherine Mochal. The author below has no conflict of interest. Ann Rashmir-Raven.

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