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The low molecular weight fraction of human serum albumin upregulates COX2, prostaglandin E2, and prostaglandin D2 under inflammatory conditions in osteoarthritic knee synovial fibroblasts

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ABSTRACT

Background: The ability to decrease inflammation and promote healing is important in the intervention and management of a variety of disease states, including osteoarthritis of the knee (OAK). Even though cyclooxygenase 2 (COX2) has an established pro-inflammatory role, evidence suggests it is also critical to the resolution that occurs after the initial activation phase of the immune response. In this study, we investigated the effects of the low molecular weight fraction of 5% human serum albumin (LMWF-5A), an agent that has proven to decrease pain and improve function in OAK patients after intra-articular injection, on the expression of COX2 and its downstream products, prostaglandins (PGs).

Methods: Fibroblast-like synoviocytes from the synovial membrane of OAK patients were treated with LMWF-5A or saline as a control with or without the addition of interleukin-1β (IL-1β) or tumor necrosis factor α (TNFα) to elicit an inflammatory response. Cells were harvested for RNA and protein at 2, 4, 8, 12, and 24 h, and media was collected at 24 h for analysis of secreted products. *COX2* mRNA expression was determined by qPCR, and COX2 protein expression was determined by western blot analysis. Levels of prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2) in the media were quantified by competitive ELISA.

Results: In the presence of either IL-1β or TNFα, LMWF-5A increased the expression of both *COX2* mRNA and protein, and this increase was significant compared to that observed with IL-1β- or TNFα-stimulated, saline-treated cells. Downstream of COX2, the levels of PGE2 were increased only in TNFα-stimulated, LMWF-5A-treated cells; however, in both IL-1β- and TNFα-stimulated cells, LMWF-5A increased the release of the anti-inflammatory prostaglandin PGD2.

Conclusion: LMWF-5A appears to trigger increased anti-inflammatory PG signaling, and this may be a primary component of its therapeutic mode of action in the treatment of OAK.

Abbreviations: OAK, osteoarthritis of the knee; COX, cyclooxygenase; LMWF-5A, molecular weight fraction of human serum albumin under 5000 Da; PG, prostaglandin; IL, interleukin; TNF, tumor necrosis factor; qPCR, quantitative real-time polymerase chain reaction; PGE2, prostaglandin E2; PGD2, prostaglandin D2; OA, osteoarthritis; NSAIDs, non-steroidal anti-inflammatory drugs; PGH2, prostaglandin H2; HSA, human serum albumin; PBMCs, peripheral blood mononuclear cells; PVDF, polyvinylidene fluoride; HSF-OAs, human synovial fibroblasts from patients with osteoarthritis; DMEM/F12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; FBS, fetal bovine serum; $\Delta\Delta C_T$, comparative threshold cycle; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean; LOD, limit of detection; hMSCs, human mesenchymal stem cells; 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; NF-κB, nuclear factor-κB; PPARγ, peroxisome proliferator-activated receptor γ

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1. Background

Osteoarthritis (OA), the most common form of arthritis, affects millions of people in the US [1] and worldwide. It is associated with symptoms of joint pain, stiffness, and swelling and risk factors of age, obesity, injury, gender, and genetic predisposition [2]. The standard treatments for OA include over-the-counter medications, such as acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), and intra-articular injections of steroids or hyaluronic acid [3]. This debilitating condition lessens the quality of life of a large percent of the aging population and incurs significant costs to society due to disability, outpatient treatments, and hospitalization for joint replacement [4,5]. In addition, the prevalence of arthritis and its associated costs are projected to rise in upcoming years as a result of the increase in the overall age and obesity of the world's population [6].

The knee is one of the most common joints affected by OA, along with the hands and hips [1]. Osteoarthritis of the knee (OAK) is physically characterized by a progressive loss of articular cartilage, a narrowing of the joint space, and the development of osteophytes [7]. The resulting and sustained mechanical stress found in OAK drives cartilage degeneration and tissue damage, promoting a chronic inflammatory environment [8,9]. Specifically, damaged cells release molecules called damage-associated molecular patterns that act to promote an innate immune response and the production of pro- and anti-inflammatory cytokines [10]. Pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (TNFα), have been found in early OA lesions and synovial fluid [8,11]. These cytokines promote further joint degeneration by inducing the expression of proteolytic enzymes, amplifying the continuous cycle of inflammation in OAK [12].

An important target of anti-inflammatory drugs, NSAIDs in particular, are the prostaglandin-endoperoxide synthase or cyclooxygenase (COX) enzymes [13]. There are two isoforms of COX; COX1 is constitutively expressed and is responsible for the regulation of normal physiological processes, while COX2 is induced under conditions of inflammation and is thought to regulate the inflammatory process [14]. COX enzymes catalyze the conversion of arachidonic acid, which is liberated from cell membranes by phospholipase A2, into prostaglandin H2 (PGH2). PGH2 then acts as a substrate for a number of synthases and isomerases to produce a panel of eicosanoids, including prostaglandins, prostacyclin, and thromboxane. These products can then bind to specific membrane receptors in an autocrine or paracrine fashion. In this manner, they exert a wide array of various and even opposing effects. Their effects depend on a number of factors in the cellular environment, *e.g.* their relative levels, the isoform of the receptor to which they bind as well as the differential expression of the receptors. For example, during the immune response, there is a shift in the relative levels of prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2), as PGE2 is increased during the initial inflammatory cascade for insult clearance and PGD2 is increased during the active event of inflammatory resolution and healing [15]. Furthermore, COX2-specific inhibitors have been shown to inhibit both phases of the immune response and to prolong inflammation [16–18], revealing the importance of COX2 in healing as well as in the initial signaling, which results in pain and swelling.

In a recent clinical trial, pain associated with OAK was significantly decreased with intra-articular injections of the low molecular weight fraction of 5% human serum albumin (HSA) under 5000 Da (LMWF-5A) [19]. HSA has been used to increase blood plasma volume in critically ill patients for decades [20], and the <5000 Da components of commercial HSA preparations have been shown to inhibit the release of inflammatory cytokines, specifically TNFα, by peripheral blood mononuclear cells (PBMCs) *in vitro* [21]. Considering that LMWF-5A decreases pain and promotes mobility in OAK patients *in vivo* and is anti-inflammatory *in vitro,* we sought to determine the effect of LMWF-5A on the COX2 pathway.

OA is induced in animal models via genetic modification, surgical alteration to the joint, or intra-articular injection of agents such as pro-inflammatory mediators [22]. LMWF-5A would likely act as a pro-inflammatory, OA-inducing agent if injected into animal models since LMWF-5A originates from a human source and may lead to an antigenic response due to its foreign nature. Due to this fact, *in vivo* characterization LMWF-5A is not feasible in animal models, and hence, the Federal Drug Administration has excluded the requirement for animal model studies of LMWF-5A safety and efficacy. However, several clinical trials have demonstrated safety and efficacy in humans suffering from OAK [19]. Thus, our working systems include *in vitro* analyses of primary cells from patients with OAK and *in vivo* analyses of patient outcomes from past and ongoing clinical trials.

LMWF-5A inhibits cytokine release *in vitro* [21,23], and clinical trial results support an anti-inflammatory mode of action *in vivo* [19]. The mode of action of NSAIDs is to inhibit COX2 enzymatic function and subsequent PG production [13], thus, we hypothesize that LMWF-5A may function by a similar mechanism. In this study, we examined COX2 levels and prostaglandin production upon LMWF-5A treatment in primary synovial fibroblasts isolated from the knees of OA patients.

2. Methods

2.1. LMWF-5A production

LMWF-5A was produced at Ampio Pharmaceuticals (Englewood, CO) as previously described [19]. Briefly, 5% HSA (Octapharma, Hoboken, NJ) was subjected to tangential flow filtration through a PVDF membrane with a 5000 Da molecular weight cutoff. The <5000 Da fraction was aseptically filled into glass vials, sealed, and stored in the dark at room temperature.

2.2. Cell culture

Human synovial fibroblasts from patients with osteoarthritis (HSF-OA; purchased from Asterand, Detroit, MI) were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; ThermoFisher Scientific, Waltham, MA) containing 20% fetal bovine serum (FBS). Before plating for experiments, the cells were fed every two to three days with media containing 10% FBS for two media changes and used between passage 8 and 11.

For the time course of COX2 mRNA and protein expression, HSF-OAs (1×10^5 cells) were plated in each well of a 24-well plate in 500 μL DMEM/F12 containing 10% FBS and incubated at 37 °C and 5% CO_2. 500 μL saline or LMWF-5A ± IL-1β (R&D Systems, Minneapolis, MN) or TNFα (ThermoFisher Scientific, Waltham, MA) (final concentration of 10 ng/mL) were added, and the cells were treated for 2, 4, 8, 12, or 24 h before harvesting for RNA and protein. For the evaluation of prostaglandin release, HSF-OAs were plated and treated as above. After 24 h, the media was collected, treated if necessary (as described below), and stored frozen until use.

2.3. Quantitative real-time PCR (qPCR) for COX2

RNA from all time-points was isolated from the treated HSF-OAs using the miRNeasy kit (Qiagen, Valencia, CA), with 1 min of vortexing for homogenization. 0.5 μg of total RNA was then reverse

transcribed into cDNA with the Qiagen QuantiTect kit. qPCR was then performed in duplicate using SYBR Green I Master Mix (Roche Diagnostics, Indianapolis, IN), a RT^2 qPCR primer assay for *COX2* (Qiagen), and a QuantiTect primer assay for 18S rRNA (Qiagen) on a Roche 480 Lightcycler. Relative gene expression was calculated using the comparative threshold cycle ($\Delta\Delta C_T$) method versus a 0 h untreated control, with normalization to 18S rRNA expression.

2.4. COX2 western blot analysis

HSF-OAs, as plated above, were lysed in 50 μL lysis buffer (Qproteome Mammalian Protein kit; Qiagen) according to manufacturer's instructions and centrifuged at 12000 × *g* at 4 °C for 10 min to remove the cellular debris. Lysates from all time-points were prepared for western blot analysis by boiling in Bolt Reducing Buffer and Bolt LDS Sample Buffer (ThermoFisher Scientific, Waltham, MA). The lysates were separated by SDS-PAGE (8%) and subjected to western blot analysis using an anti-COX2 rabbit monoclonal primary antibody (1:1000, ab62331; Abcam, Cambridge, MA) and a goat anti-rabbit IgG secondary antibody (1:10000, Cat# 7074P2, Cell Signaling, Danvers, MA). The COX2 protein levels were normalized to α-tubulin after stripping and reprobing with Reblot Plus (Millipore, Billerica, MA) and a horseradish peroxidase-conjugated α-tubulin antibody (1:5000, DM1A, Cat# 12351S, Cell Signaling, Danvers, MA), respectively.

2.5. Prostaglandin enzyme-linked immunosorbent assays (ELISAs)

Conditioned media for the two prostaglandin assays used in this study was harvested from the same well, split into two and processed as necessary for either PGE2 or PGD2 analysis. The levels of PGE2 in the media were analyzed using a competitive Prostaglandin E2 ELISA kit (Abcam, Cambridge, MA) following the manufacturer's protocol. The levels of PGD2 in the media were analyzed using a competitive Prostaglandin D2-MOX EIA kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions. Notably, with this kit, the PGD2 in the sample is stabilized upon a 30-min incubation at 60 °C with a methyloximating reagent immediately following sample collection.

2.6. IL-1β ELISA

Cell culture media was collected from OA synovial fibroblast cultures 24 h after stimulation with 10 ng/mL IL-1β in the presence of saline or LMWF-5A and diluted to fall within the range of the standard curve. IL-1β was quantified by sandwich ELISA (Abcam, Cambridge, MA).

2.7. Statistical analysis

All graphs and figures represent four independent experiments with the exception of Supplemental Fig. 1, which represents three independent experiments. Graphs represent the mean ± standard deviation (SD). SAS (Statistical Analysis System) 9.3 software (SAS Institute Inc., Cary, NC) was used for the statistical analysis; p-values were calculated using a student's paired two-tailed *t*-test, and $p < 0.05$ was accepted as significant.

3. Results

3.1. LMWF-5A increases COX2 in cytokine-stimulated OA synovial fibroblasts

Using primary synovial fibroblasts isolated from the knee synovial membrane of patients with OA, we tested our hypothesis,

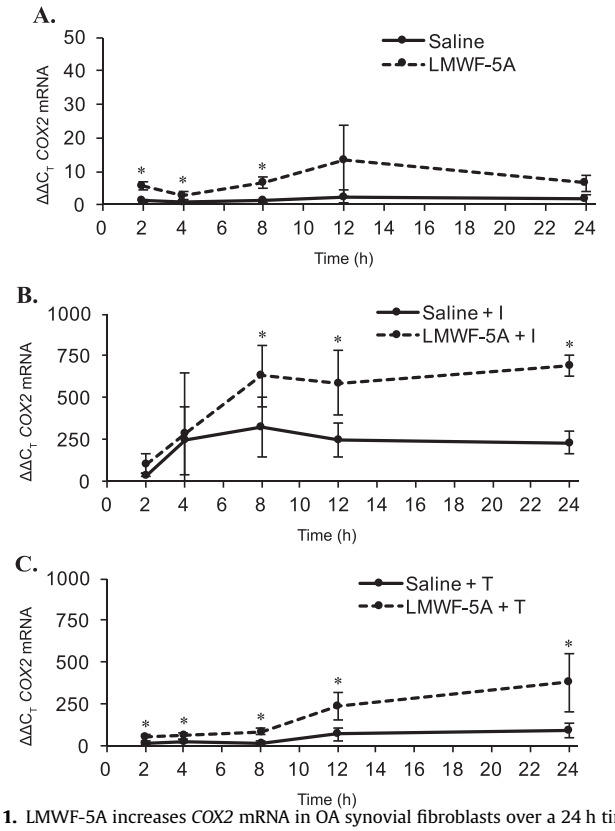

Fig. 1. LMWF-5A increases *COX2* mRNA in OA synovial fibroblasts over a 24 h time course. OA synovial fibroblasts were cultured in the presence of LMWF-5A or saline alone (A), LMWF-5A or saline with 10 ng/mL IL-1β (B), or LMWF-5A or saline with 10 ng/mL TNFα (C) for up to 24 h. Total RNA was harvested 2, 4, 8, 12, and 24 h post-treatment, and qPCR was performed to quantify the total *COX2* mRNA and 18 S rRNA expression. Using the $\Delta\Delta C_T$ method, relative fold changes were quantified and normalized to untreated OA synovial fibroblasts. The normalized fold-change (mean ± SD) is shown. * indicates significantly increased *COX2* mRNA when compared to the saline control at that time point ($p < 0.05$; n = 4).

that one of the mechanisms of action of LMWF-5A is similar to that of NSAIDs, by quantifying *COX2* mRNA by qPCR and COX2 protein by western blotting over a 24 h time course. HSF-OAs incubated with LMWF-5A without cytokine showed an increase in *COX2* mRNA of 3 to 13-fold when normalized back to untreated controls over a 24 h time course (Fig. 1A; $p < 0.05$ for LMWF-5A at 2, 4, and 8 h). HSF-OAs treated with saline and then stimulated with IL-1β or TNFα showed an induction in *COX2* mRNA of 19 to 327-fold when normalized back to untreated cells over a 24 h time period (Fig. 1B and C). Surprisingly, over the same time course, HSF-OAs stimulated with IL-1β or TNFα induced *COX2* mRNA expression 52 to 692-fold, an additional ~1.2 to ~4.0-fold higher, in the presence of LMWF-5A (Fig. 1B and C; $p < 0.05$ for LMWF-5A + IL-1β at 8, 12, and 24 h and LMWF-5A + TNFα at 2, 4, 8, 12, and 24 h). Interestingly, when COX2 protein was measured by western blot, significant fold increases in COX2 protein were only observed when OA synovial fibroblasts were stimulated with either IL-1β or TNFα in the presence of LMWF-5A (Fig. 2). There was no difference in COX2 protein without IL-1β or TNFα exposure in OA synovial fibroblasts treated with either LMWF-5A or saline in contrast to the observed *COX2* mRNA dynamics (Fig. 2A). However, IL-1β-stimulated, LMWF-5A-treated cells displayed significantly higher levels of COX2 protein between 8 and 24 h (Fig. 2B; $p < 0.05$ for LMWF-5A + IL-1β at 8, 12, and 24 h). When stimulated with TNFα in the presence of LMWF-5A, COX2 protein was significantly increased over TNFα-stimulated, saline-treated HSF-OAs at 12 h (Fig. 2C; $p < 0.05$ for LMWF-5A + TNFα at 12 h). Thus, in OA synovial fibroblasts, both *COX2* mRNA and COX2 protein significantly

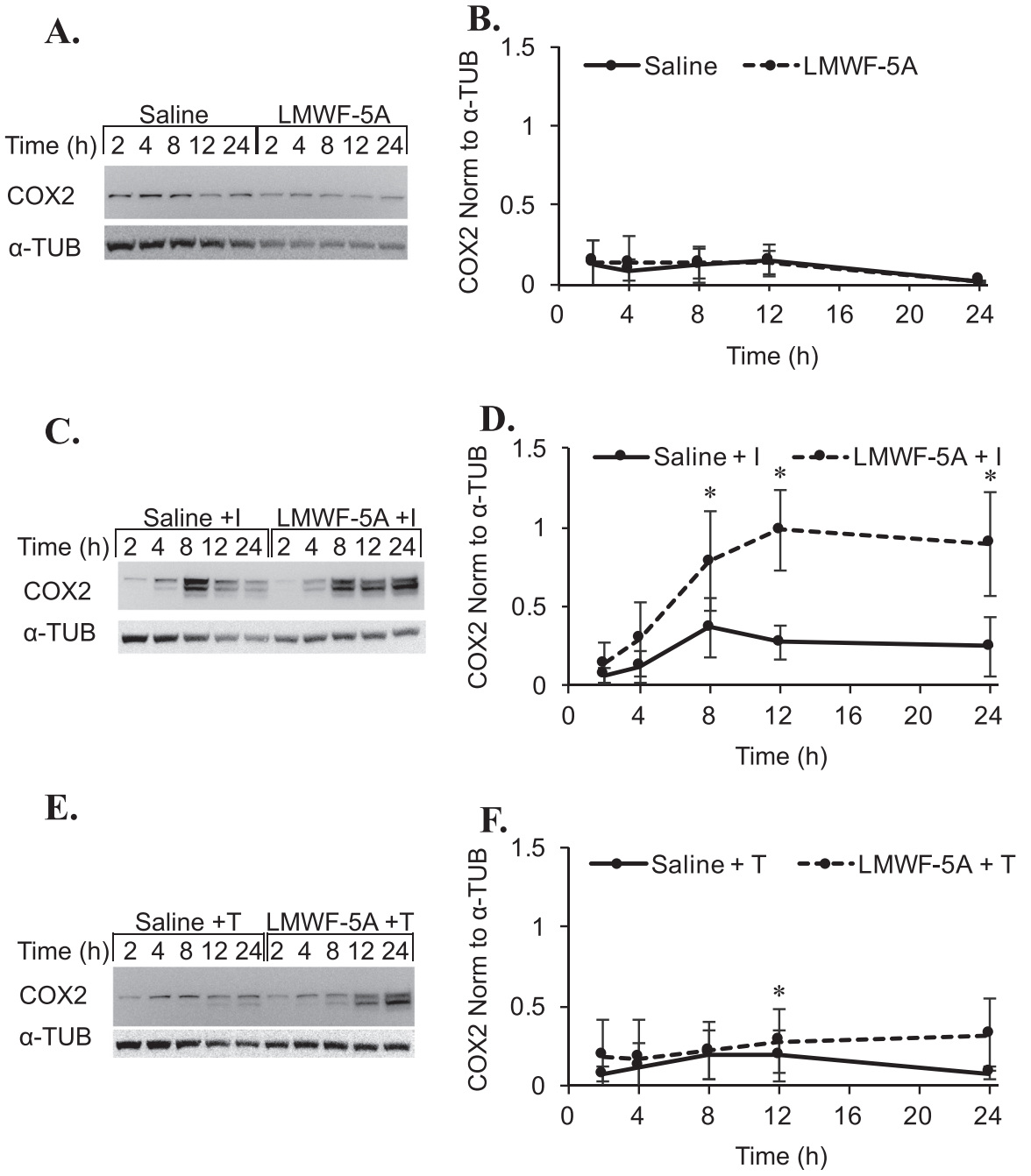

Fig. 2. LMWF-5A increases COX2 protein in OA synovial fibroblasts when co-stimulated with pro-inflammatory cytokines. Western blots of protein lysates from OA synovial fibroblasts were probed with antibodies against COX2 and the loading control α-Tubulin (A, C, E). Relative band densities were graphed over time (B, D, F). OA synovial fibroblasts were left unstimulated (A, B), stimulated with 10 ng/mL IL-1β (C, D), or 10 ng/mL TNFα (E, F) in the presence of saline or LMWF-5A over a 24 h time course, and protein lysates were prepared at 2, 4, 8, 12, and 24 h. The normalized COX2 protein level (mean ± SD) is shown. The multiple bands observed with COX2 immunoblotting may be due to the multiple glycosylation states of COX2 [33]. * indicates significantly increased *COX2* mRNA when compared to the saline control at that time point ($p < 0.05$; $n=4$).

increase only when these cells are stimulated with IL-1β or TNFα in the presence of LMWF-5A.

3.2. *LMWF-5A increases prostaglandin release from cytokine-stimulated OA synovial fibroblasts*

Considering that COX2 expression is increased under inflammatory conditions in the presence of LMWF-5A, we next investigated downstream products of COX2 in this system. We focused on two products, PGE2 and PGD2. PGE2 has been implicated in the initial phase of the innate immune response, the clearance of the insult, as well as in the promotion of tissue regeneration [24]. PGD2 has been linked to the second phase, resolution and healing [15]. As described above, OA synovial fibroblasts were treated with saline as a control or LMWF-5A with or without IL-1β or TNFα. The amount of each prostaglandin secreted into the media was determined with a specific competitive ELISA after 24 h of treatment (Fig. 3).

The level of PGE2 in the media of cells treated solely with saline or LMWF-5A was below the limit of detection (LOD) of this assay (39.1 pg/mL); however, when stimulated with cytokine, OA synovial fibroblasts produced detectable levels of PGE2. Upon treatment with

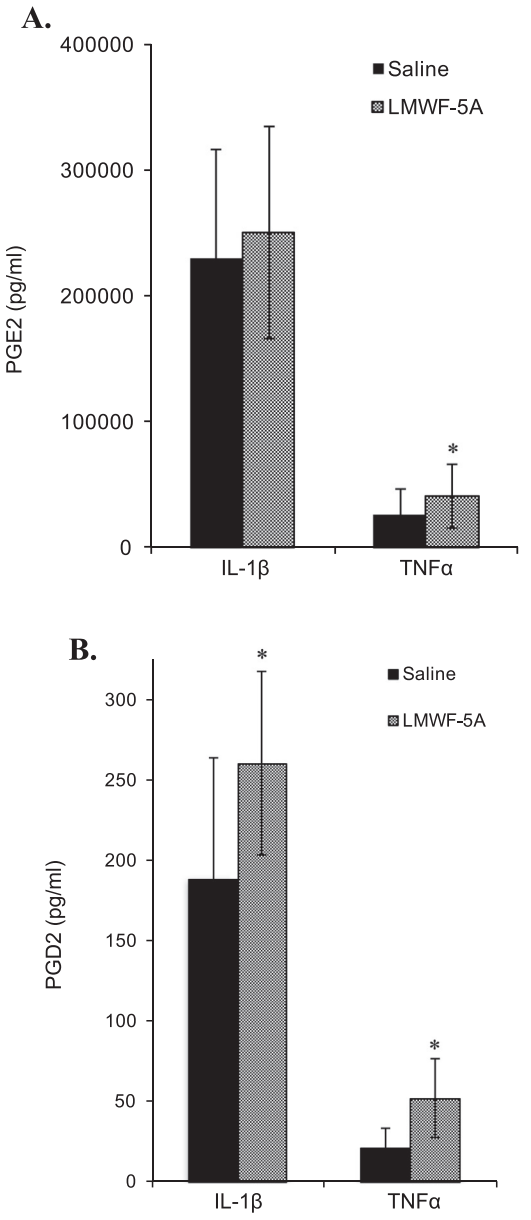

Fig. 3. LMWF-5A affects prostaglandin release by cytokine-stimulated OA synovial fibroblasts. Cell culture media was collected from OA synovial fibroblast cultures 24 h after stimulation with either 10 ng/mL IL-1β or TNFα in the presence of saline or LMWF-5A. PGE2 (A) and PGD2 (B) were quantified by competitive ELISA. The mean concentration ± SD for four independent experiments were graphed, and * indicates a significant increase ($p < 0.05$) in PG in the media when compared to the corresponding saline control.

IL-1β or TNFα for 24 h under saline conditions, the media contained 231,000 pg/mL and 26,300 pg/mL of PGE2, respectively (Fig. 3A). When the cells were stimulated with IL-1β in the presence of LMWF-5A, the concentration of PGE2 in the media (251,000 pg/mL) was not significantly different from that found in the media of IL-1β-stimulated cells in the presence of saline (Fig. 3A). In contrast, the level of PGE2 in the media of TNF-stimulated, LMWF-5A-treated cells was increased by 58% (41,500 pg/mL, $p < 0.05$) compared to that found in the media of TNF-stimulated, saline-treated cells (Fig. 3A).

Similar to PGE2, unstimulated OA synovial fibroblasts exhibited no detectable release of PGD2 (LOD = 2 pg/mL). Upon addition of IL-1β or TNFα to saline-treated cells, the concentration of PGD2 in the media increased to 189 pg/mL and 26.1 pg/mL, respectively (Fig. 3B). Interestingly, under these conditions, LMWF-5A significantly increased the release of PGD2 to 261 pg/mL with IL-1β stimulation and 52.4 pg/ml with TNFα stimulation, signifying 38% and 101% increases, respectively, ($p < 0.05$, Fig. 3B). It is important to note that PGE2 is a stable molecule, while PGD2 is unstable and must be chemically modified to prevent its degradation. Thus, in this experiment, the level of PGE2 reflects the accumulation of PGE2 over the 24 h time course, and the level of PGD2 represents a snapshot of the PGD2 release at the time of sample collection.

4. Discussion

HSA has been used for fluid resuscitation and, more recently, for chronic liver and renal failure [20], and the fraction of 5% HSA under 5000 Da, LMWF-5A, has been shown to inhibit the release of inflammatory cytokines [21,23]. Clinical trials, in which the efficacy and safety of LMWF-5A as a treatment for osteoarthritis of the knee were tested, have shown that injection of LMWF-5A into the knee joint improves function and decreases pain in osteoarthritic knees, implicating an anti-inflammatory mode of action *in vivo* [19]. Other anti-inflammatory drugs, such as NSAIDs, have long been used to treat pain and swelling in osteoarthritis [3]. Thus, we postulated that LMWF-5A may have a mechanism of action that is similar to that of NSAIDs, *i.e.*, blocking the enzymatic function of COX2 and the subsequent downstream production of PGs [13]. In this study, we tested this hypothesis with primary human OA synovial fibroblasts, allowing us to address one of the relevant cell types within the osteoarthritic knee. Here we report that unexpectedly, LMWF-5A super-induces COX2 when HSF-OAs are stimulated with either IL-1β or TNFα. We also observed a more pronounced response in IL-1β-stimulated, LMWF-5A-treated HSF-OAs as compared to TNFα-stimulated, LMWF-5A-treated cells. Several cytokines and chemokines, including IL-1β and TNFα, have been implicated in the progression of OA; however, important differences exist between IL-1β and TNFα with respect to OA. Increased IL-1β levels are found in OA sera compared to normal sera [25], and synovial membrane and cartilage samples from patients with OA show higher levels of IL-1β-converting enzyme, which is required to process the precursor form of IL-1β into mature cytokine [26]. In a mouse model of arthritis, IL-1 blockade prevents further disease progression, whereas inhibition of TNFα only decreases inflammation within the joint [27]. TNFα is significantly increased in OA synovial fluid when compared to normal synovial fluid but is absent in OA sera [25]. Our observed differences in the effects of IL-1β versus TNFα stimulation in the presence of LMWF-5A were pronounced with respect to COX2. Induction of COX2 in IL-1β-stimulated, LMWF-5A-treated cells was much higher compared to TNFα-stimulated, LMWF-5A-treated cells; however, quantification of PGE2 release from IL-1β-stimulated cells in the presence of LMWF-5A showed no significant difference. It was only under TNFα-stimulated, LMWF-5A-treated conditions that a significant increase in PGE2 was observed. It is important to note that the relative levels of PGE2 were 10-fold higher under IL-1β-stimulated conditions.

Considering that COX2 expression and PGE2 production are either the same or increased with LMWF-5A under pro-inflammatory cytokine conditions, one may expect that LMWF-5A injection into the OA knee would elicit a localized inflammatory response. Eliciting an inflammatory response as a treatment has been coined as prolotherapy, which is characterized by redness, swelling, and pain following injections of prolotherapeutics, such as hypertonic dextrose and morrhuate sodium [28]. The premise of prolotherapy is to elicit an inflammatory response that acts to trigger resolution and healing signaling cascades. Based on clinical trial data, LMWF-5A does not cause a localized inflammatory

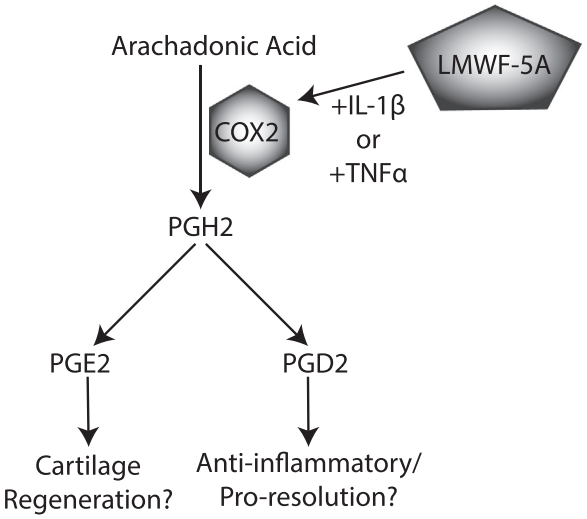

Fig. 4. Proposed mechanism of action for LMWF-5A with respect to the COX2 pathway. In the inflammatory environment of OAK, LMWF-5A-treated synovial fibroblasts increase their expression of COX2 and the downstream prostaglandins PGE2 and PGD2. Importantly, no inflammatory responses have been observed in either OAK patients or cell culture upon intra-articular injection or treatment with LMWF-5A. We hypothesize that LMWF-5A increases COX2 and downstream PGE2 release from synovial fibroblasts into the synovial fluid, inducing regeneration of cartilage, as they have been implicated in promoting chondrogenesis and tissue regeneration. Furthermore, we have previously shown that LMWF-5A increases chondrocyte condensation in stem cells. Additionally, increased PGD2 release from synovial fibroblasts observed upon LMWF-5A treatment may trigger resolution of inflammation and healing via NF-κB inhibition and PPARγ activation.

response *in vivo*, as patients do not experience joint swelling but do experience rapid pain relief that persists 12 weeks post-injection [19]. Additional evidence that LMWF-5A does not elicit an acute inflammatory response is that PBMCs stimulated with lipopolysaccharide release significantly less TNFα into the medium when co-treated with LMWF-5A [21], and treatment of HSF-OAs with LMWF-5A and either IL-1β or TNFα does not increase IL-1β release into the media (Supplemental Fig. 1).

Because we do not observe hallmarks of an acute inflammatory response either *in vivo* or *in vitro*, LMWF-5A may circumvent this response and directly initiate healing and regeneration in the knee. Recent evidence has shown that inhibiting PGE2 degradation, thus increasing the tissue PGE2 concentration, potentiates multi-tissue regeneration and increases hematopoiesis and bone marrow stem cell fitness [24]. Fibroblasts found in the synovial fluid are closely related to bone marrow stem cells [29] and may be a source of resident stem cells within the knee. Previously, we have shown that LMWF-5A drives chondrocyte condensation in human mesenchymal stem cells (hMSCs) [30], and inhibition of *COX2* disrupts hMSC chondrogenesis [31]. Thus, injection of LMWF-5A may influence multiple cell populations within the knee, synoviocytes, resident stem cells, and chondrocytes, to regenerate damaged cartilage through upregulation of COX2 and PGE2. Furthermore, we observed a significant increase in PGD2 release from LMWF-5A-treated HSF-OAs under IL-1β and TNFα conditions. Increased PGD2 may trigger an anti-inflammatory/pro-resolution cascade, as it spontaneously undergoes non-enzymatic dehydration and is converted into 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), a cyclopentenone PG that has been shown to be immuno-modulatory and anti-inflammatory by its ability to inhibit NFκB signaling and cytokine release and to act as an agonist of PPARɣ [32]. We attempted to measure 15d-PGJ2 using a commercially available competitive ELISA but found that LMWF-5A interfered with quantification, resulting in artificially increased values. Nonetheless, our data clearly shows that LMWF-5A significantly increases anti-inflammatory PGD2 release. Since increased release of PGs may be a key aspect of the therapeutic action of LMWF-5A and because NSAIDs inhibit the production of all subclasses of PGs due to the inhibition of upstream COX2 enzymatic action, these results have influenced a current clinical trial evaluating LMWF-5A, resulting in the exclusion of NSAID use by trial participants (NCT02556710).

5. Conclusion

In summary, we propose a mechanism in which LMWF-5A increases the level of COX2 and its downstream PGs in an inflammatory environment, promoting resolution of inflammation and healing as well as cartilage regeneration (Fig. 4). Because PGE2 and other PGs act in both paracrine and autocrine fashions, these molecules can be produced by one cell type in the knee but exert their effects on another. Our data provide important insight into the effect of LMWF-5A on HSF-OAs; however, several other cell populations reside within the knee and influence the overall inflammatory status and disease progression. We are currently exploring the regulation of COX2 and PG release in response to LMWF-5A in several other relevant cell types and their combination to gain an understanding of their interplay in the OA knee environment.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.015.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.015.

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