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Lactoferricin mediates Anti-Inflammatory and Anti-Catabolic Effects *via* Inhibition of IL-1 and LPS Activity in the Intervertebral Disc†

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Abstract

The catabolic cytokine interleukin-1 (IL-1) and endotoxin lipopolysaccharide (LPS) are well-known inflammatory mediators involved in degenerative disc disease, and inhibitors of IL-1 and LPS may potentially be used to slow or prevent disc degeneration *in vivo*. Here, we elucidate the striking anti-catabolic and anti-inflammatory effects of bovine lactoferricin (LfcinB) in the intervertebral disc (IVD) *via* antagonism of both IL-1 and LPS-mediated catabolic activity using *in vitro* and *ex vivo* analyses. Specifically, we demonstrate the biological counteraction of LfcinB against IL-1 and LPS-mediated proteoglycan (PG) depletion, matrix-degrading enzyme production and enzyme activity in long-term (alginate beads) and short-term (monolayer) culture models using bovine and human nucleus pulposus (NP) cells. LfcinB significantly attenuates the IL-1 and LPS-mediated suppression of PG production and synthesis, and thus restores PG accumulation and pericellular matrix formation. Simultaneously, LfcinB antagonizes catabolic factor mediated induction of multiple cartilage-degrading enzymes, including MMP-1, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5, in bovine NP cells at both mRNA and protein levels. LfcinB also suppresses the catabolic factor-induced stimulation of oxidative and inflammatory factors such as iNOS, IL-6, and toll-like receptor-2 (TLR-2) and TLR-4. Finally, the ability of LfcinB to antagonize IL-1 and LPS-mediated suppression of PG is upheld in an *en bloc* intradiscal

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microinjection model followed by *ex vivo* organ culture using both mouse and rabbit IVD tissue, suggesting a potential therapeutic benefit of LfcinB on degenerative disc disease in the future.

Keywords

Lactoferricin; Interleukin-1; Lipopolysaccharide; Intervertebral disc degeneration; Anti-Inflammation; Anti-catabolic effect

Introduction

Chronic low back pain, a common public health problem, has become a serious social concern in modern societies. While the etiology of low back pain is likely multi-factorial, recent studies have demonstrated that it is closely associated with degeneration of the intervertebral disc (IVD) (Luoma et al., 2000; Luoma et al., 2004). Disc degeneration results from an imbalance between degradation (catabolism) and synthesis (anabolism) of extracellular matrix (ECM) components by chondrocytes residing in the nucleus pulposus (NP) (Iatridis et al., 1997; Nomura et al., 2001). Via an upregulation of molecules such as pro-inflammatory cytokines and catabolic growth factors, homeostasis of the ECM shifts toward a degenerative state (Lee et al., 2009). For example, a relative increase in destructive enzymes, including matrix metalloproteases (MMPs) and a disintegrin-like and metalloprotease with thrombospondin motifs (ADAMTS family, aggrecanases), may alter the normal balance within the disc and result in degeneration (Crean et al., 1997; Le Maitre et al., 2004; Le Maitre et al., 2007b). Recently, biological treatments capable of preventing disc degeneration by promoting ECM repair and regeneration have been considered (An et al., 2003a; An et al., 2003b; Masuda, 2008), and a greater understanding of the catabolic pathways involved in this process would help to identify potential prophylactic or regenerative biological treatments in the future.

Previous studies have linked IVD degeneration with a variety of pro-inflammatory cytokines, growth factors, and metabolites, including interleukin-1 (IL-1) (Burke et al., 2002b; Millward-Sadler et al., 2009), lipopolysaccharide (LPS) (Liu et al., 2010), tumor necrosis factor- α (TNF α) (Millward-Sadler et al., 2009), basic fibroblast growth factor (bFGF, aka FGF-2) (Ellman et al., 2008), and reactive oxygen species (ROS) (Kim et al., 2009). Among these, IL-1 and LPS are well-known pro-inflammatory mediators involved in disc degeneration. IL-1 has been shown to demonstrate the most potent deteriorative biological activity in the IVD, in part due to significant upregulation of destructive enzymes in degenerative disc tissue (Le Maitre et al., 2007a). LPS is an endotoxin found in the outer membrane of gram-negative bacteria, and has been found to play a pivotal role in inflammatory states and arthritic conditions (Bobacz et al., 2007; Iacono et al., 2010; Idogawa et al., 1998). The literature suggests a potent catabolic role of LPS on cartilage matrix homeostasis via LPS-induced, toll-like receptor (TLR)-2 and TLR-4-mediated suppression of proteoglycan (PG) synthesis and stimulation of PG degradation in a variety of cartilaginous tissues (Bobacz et al., 2007; Jasin, 1983; Kittlick and Engelmann, 1991). Therefore, inhibiting the activity of IL-1 and/or LPS via the use of antagonists may serve as a potential treatment strategy to slow cartilage degeneration and shift homeostasis away from catabolism (Hickey et al., 2003; Li et al., 2008b; Trippel et al., 2007), with the ultimate goal of retarding the process of IVD degeneration.

Bovine lactoferricin (LfcinB) is obtained by acid-pepsin hydrolysis of the N-terminal region of lactoferrin from cow's milk (Mader et al., 2007; Tsuda et al., 2002). It exerts more potent biological effects than equimolar amounts of intact lactoferrin, is cell membrane-permeable, and interacts electrostatically with negatively-charged matrix and cell surface

glycosaminoglycans, heparin and chondroitin sulfate (Baker and Baker, 2009; Gifford et al., 2005). The anti-inflammatory, anti-viral, anti-bacterial, anti-oxidant and anti-cancer properties of LfcinB have been reported in a variety of tissues *in vitro* (Daidone et al., 2011; Gifford et al., 2005). More recently, the anti-oxidative and anabolic effect of LfcinB has been reported, suggesting a possible chondroprotective biological role in both articular cartilage and bovine disc cells (Afonso et al., 2007; Henrotin et al., 2003). However, the precise molecular mechanisms by which LfcinB exerts chondroprotective and anti-inflammatory effects in the IVD have yet to be established.

In the present study, we analyze the potential for anabolic and anti-catabolic effects mediated by LfcinB on IVD homeostasis by assessing its anti-inflammatory effects in the presence of IL-1 and LPS in human, rabbit, mouse, and bovine disc tissue. We begin to unravel the complex intracellular signaling cascades that mediate LfcinB's inhibition of IL-1 and LPS-mediated effects via regulation of TLRs in the bovine IVD, and our *in vitro* results are corroborated by *ex vivo* histological analyses using mice discs, revealing a significant therapeutic potential of LfcinB to retard or reverse the progression of IVD degeneration.

MATERIALS AND METHODS

IVD cell isolation and culture

Bovine coccygeal discs were dissected en bloc, and the NP portion of each disc was separated sharply. The cells were released by enzymatic digestion in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) culture medium with sequential treatments of 0.2% pronase and 0.025% collagenase P, as previously described (Gruber et al., 2006; Im et al., 2003). Alginate beads and monolayers were prepared for long-term (21 days) and short-term studies (1–2 days), respectively (Im et al., 2008; Im et al., 2007; Im et al., 2003; Kim et al.; Li et al., 2008a).

For long-term alginate bead culture (21 days), isolated disc cells were resuspended in 1.2% alginate, and beads were formed by drop-wise addition into a CaCl₂ solution as previously described (Gruber et al., 2006; Hauselmann et al., 1996; Im et al., 2003). Beads were cultured at 8 beads/well in 24-well plates in 1 mL/well of DMEM/Ham's F-12 medium supplemented with 1% mini-ITS (insulin–transferrin–selenium) (Gruber et al., 2006; Im et al., 2003). Cells were treated with 10 µg/mL LfcinB (Biosynthesis, Lewisville, Texas), 1 ng/mL IL-1 (National Cancer Institute, Frederick, MD), 1 µg/mL LPS (Sigma-Aldrich, St Louis, MO) and LfcinB plus IL-1 or LPS at the above concentrations. Media were changed every other day for a 21-day period before dimethylmethylene blue (DMMB) assay, as described previously (Gruber et al., 2006; Kim et al., 2010; Kim et al., 2011a; Li et al., 2008a; Loeser et al., 2005).

For short-term monolayer cultures, isolated NP cells from either bovine tails or human lumbar spine discs (Gift of Hope Organ and Tissue Donor Network, Elmhurst, IL, obtained with IRB approval (Daidone et al., : 08082803-IRB01)) were counted and plated onto 12-well plates at 8×10^5 cells/cm² as previously described (Im et al., 2007; Im et al., 2003). The cells were treated with synthesized peptide 100 µg/mL of LfcinB (Biosynthesis, Lewisville, Texas) and/or 10 ng/mL IL-1 or 10 µg/mL LPS, in 1 mL per well of serum-free medium for 24 hours at 37° under 5% CO₂. Supernatants were collected 24 hours after the initiation of each treatment and subjected to western blotting analyses. Cells were treated for 24 hours before total RNA harvesting.

Dimethylmethylene Blue assay for Proteoglycan Production and DNA Assay for Cell Numbers

At day 21 of culture, the alginate beads were collected and processed for PG assays using the DMMB assay, as previously described (Gruber et al., 2006). The PG levels measured in the cell-associated matrix (CM) were quantified per DNA to give the total amount of PGs produced and retained in the alginate beads per cell (Im et al., 2003; Loeser et al., 2005). Using PicoGreen (Molecular Probes, Carlsbad, CA), cell numbers were determined by assay of total DNA in cell pellets, as previously described (Gruber et al., 2006; Im et al., 2003; Loeser et al., 2005)

Cell survival assay

Cell survival was measured as previously described (Loeser et al., 2005), using Calcein AM to stain live cells and ethidium bromide homodimer 1 to stain dead cells. These reagents were obtained from Molecular Probes (Eugene, OR). Survival was measured after 7, 14, and 21 days of culture. At least 100 cells were counted in triplicate for each data point.

³⁵S-sulfate Incorporation into Newly-Synthesized Proteoglycans

On day 7 of culture in alginate, culture medium was removed and replaced by fresh medium. One hour later, this medium was replaced with fresh medium containing [³⁵S]-sulfate at 20 μ Ci/mL (Amersham Corp, Arlington Heights, IL). After incubation for 4 hours at 37°, the labeling medium was removed and the beads were rinsed twice in cold 1.5 mM SO₄ washing media. Beads were dissolved to separate out the CM and digested with papain (20 μ g/mL in 100 mM sodium acetate, 50 mM EDTA, pH 5.53) at 60°C for 16 hours. Sulfate incorporation into PGs was measured using the Alcian blue precipitation method (Loeser et al., 2000).

Particle Exclusion Assay for Matrix Assessment

The cells with their pericellular matrix were visualized using a particle exclusion assay, as previously described (Hauselmann et al., 1996; Knudson, 1993). Briefly, after day 21 of culture in alginate, the beads were solubilized with 55 mM sodium citrate (pH 6.8). The cells were pelleted by centrifugation, resuspended in DMEM, and then plated on a multi-well cell culture dish. The cells were allowed to settle and attach to the plates for 6 hours. Formalin-fixed erythrocytes were then added and allowed to settle for 10–15 min. Cells were then observed and photographed with an inverted phase-contrast microscope (Nikon, Melville, NY).

Reverse Transcription and Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRIzol reagent (Invitrogen) following the instructions provided by the manufacturer. RT was carried out with 1 μ g total RNA using the ThermoScript RT-PCR system (Invitrogen) for first-strand complementary DNA (cDNA) synthesis. For real-time RTPCR, cDNA was amplified using the MyiQ Real-Time PCR Detection System (Bio-Rad). A Ct value was obtained from each amplification curve using iQ5 Optical System Software provided by the manufacturer (Bio-Rad). Relative messenger RNA (mRNA) expression was determined using the C_T method, as detailed by the manufacturer (Bio-Rad). β -actin was used as the internal control. The primer sequences and their conditions for use are summarized in Table 1.

Western blotting

Cell and tissue lysates were prepared using modified RIPA buffer, as previously described (Li et al., 2008a; Li et al., 2008b). Total protein concentrations of cell lysates were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). An equal

amount of protein was resolved by 10% SDS-PAGE gels and transferred to a nitrocellulose membrane for western blot analyses, as described previously (Im et al., 2007). Immunoreactivity was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and the Signal Visual Enhancer system (Pierce), which magnifies the signal.

Ex vivo analysis via an intradiscal microinjection organ culture model

New Zealand white rabbits (2.5–3kg, mixed male and female) were given 1.3 mL of heparin intravenously under general anesthesia. After the heparin circulated for five minutes, the rabbits were euthanized with a lethal dose of pentobarbital to permit dissection of lumbar motion segments. The entire spine, including all osseous elements, was dissected and removed from the rabbit. LfcinB (200 µg per disc, n=6 per treatment) was then intradiscally injected *en bloc* using a 26-gauge needle (50 µL volume) (Fig. 1A). The injected disc was separated from its neighboring discs above and below using an electronic microsaw (Stryker Biotech) (Fig. 1B). The disc was maintained for 14 days in DMEM/Ham's F-12 medium supplemented with 1% mini-ITS in the presence or absence of IL-1 (100 ng/mL) or LPS (10 µg/mL).

Similar techniques were utilized using mice discs. The spine of each mouse was dissected and removed, and lumbar IVD tissue was intradiscally injected *en bloc* with LfcinB (100 µg per disc) using a 30-G needle (1.5 µL volume) followed by dissection to separate the injected discs. The discs were then maintained individually for 3 days in 1% mini-ITS supplemented serum free media in the presence or absence of IL-1 or LPS, as described above (n=6 per treatment). On the last day of organ culture, the harvested rabbit and mouse lumbar disc cells were assessed to evaluate cell viability with fluorescent microscopy using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) by modifying previously described methods (Del Carlo and Loeser, 2002; Junger et al., 2009).

Histologic Analysis

Harvested discs from the vertebral columns of rabbits or mice were fixed in 4% paraformaldehyde and decalcified in EDTA, which was changed every 5 days. The decalcified discs were paraffin embedded. Serial disc sections of exactly 5-µm thickness were obtained to prepare slides. Safranin O–fast green staining was performed to assess general morphology and the loss of PG in disc ground substance, as previously described (Muddasani et al., 2007). An unblinded investigator grouped the slides and randomly numbered them. The groups were graded by two different blinded investigators (H-JI and XL). A relative grade was assigned from 0–4, where 0 indicates no staining (complete loss of PG) and 4 indicates the most intense stain (normal disc) based on Safranin-O.

Protease activity assessments

Zymography: To analyze MMP activity, concentrated NP media were mixed with sample buffer without reducing agent or boiling, and loaded onto 1 mg/mL gelatin-containing SDS-polyacrylamide gel. After electrophoresis, the gel was washed to remove SDS by 50mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 for 1 hour at room temperature, allowing the reactivation of MMPs. The gel was stained with 0.1% Coomassie brilliant blue R250 (GE Healthcare, Piscataway, NJ, USA). Gel images were captured and the clear bands were analyzed using “ImageJ” image analysis software (www.imagej.nih.gov), and were expressed in arbitrary optical density units. Data shown are cumulative of two experiments. P-values presented as mean ± standard deviation; data without a common letter differ, $P<0.05$. **Active MMP13 ELISA:** Activity of MMP-13 was assessed by an Active MMP-13 ELISA kit (Protealimmune, InviLISA human ACT MMP-13, Cupertino, CA 95014) using conditioned media of human NP cells by following the manufacturer's instructions (standard

sandwich ELISA). A highly specific monoclonal antibody for the activated form of human MMP-13 permits the detection of a specifically active form of MMP-13 at the sensitivity level of 7 pg/mL.

Statistical Analysis

Analysis of variance was performed using StatView 5.0 software (SAS Institute, Cary, NC). P-values less than 0.05 are considered to be statistically significant in each test.

RESULTS

LfcinB counteracts the catabolic effect of IL-1 and LPS on PG accumulation in bovine NP cells

Our initial results from concentration-dependent experiments suggest that 10 μ g/mL is a minimal dose of LfcinB necessary to induce maximum biological effects in long-term alginate bead cultures of bovine NP cells (Kim et al., 2011). Therefore, in the current study, alginate beads were cultured in the presence of LfcinB (10 μ g/mL), IL-1 (1 ng/mL), LPS (1 μ g/mL), and LfcinB plus IL-1 or LPS for 21 days. Beads were subsequently disassociated and examined by a particle exclusion assay to allow visualization of ECM formation in bovine NP cells. Control bovine NP cells have a minimal amount of pericellular matrix formation [Fig. 2A (*a*)], which is almost completely suppressed in the presence of IL-1 (*b*), and to a lesser extent LPS (*c*). However, in response to LfcinB (10 μ g/mL), NP cells demonstrate a substantial increase in pericellular matrix formation (*d*). More importantly, IL-1 and LPS-suppressed pericellular matrix formation is rescued in the presence of LfcinB [Fig. 2A (*e* and *f*)]. Interestingly, despite the ability of LfcinB to antagonize the catabolic activity of IL-1 on matrix formation, IL-1 itself is capable of attenuating the anabolic effect of LfcinB in bovine NP cells, as demonstrated in Figure 2A (*d*) and (*e*).

Next, after culture in alginate for 21 days, the amount of PG in the CM was measured by DMMB assay, and normalized using DNA content. Incubation of cells with 10 μ g/mL of LfcinB significantly increases PG accumulation per cell by > 60% compared to control ($p < 0.05$, Fig. 2B). In contrast, incubation of cells with 1 ng/mL IL-1 and 1 μ g/mL LPS decreases PG deposition per cell by approximately 50% and 30%, respectively ($p < 0.05$). Importantly, when given together, LfcinB completely abolishes and reverses the IL-1- or LPS-mediated suppression of PG accumulation, upregulating PG production per cell beyond or similar to control level (no treatment) ($p < 0.05$, Fig. 2B). There is no statistically significant change in cell proliferation (assessed by total DNA) nor cell survival at the given concentrations of reagents (LfcinB, IL-1 or LPS), either individual or combined, for the current study (Fig. 2C).

In order to determine whether the increase in PG accumulation is mediated in part by a LfcinB-induced stimulation of PG synthesis, we quantitated the incorporation of 35 S-sulfate by bovine NP cells into PGs within the cell associated matrix. When NP cells are incubated with LfcinB (10 μ g/mL), PG synthesis increases by approximately 45% compared to control ($p < 0.05$). This increase remains in the presence of IL-1, suggesting that the antagonistic activity toward IL-1 by LfcinB involves an increase in PG accumulation and is mediated, at least in part, by the stimulation of PG synthesis (Fig. 2D).

To further evaluate mechanisms by which LfcinB exerts anti-catabolic activity in bovine NP cells, we assessed the effects of IL-1, LPS and LfcinB on aggrecan expression, a major component of PG in NP tissue, using real-time qPCR (Fig. 2E). For monolayer analysis, initial experiments revealed that 100 μ g/mL of LfcinB is a minimal dose demonstrating maximal effects (data not shown). Therefore, this concentration was used in all subsequent

monolayer in vitro experiments. NP cells incubated with LfcinB alone (100 µg/mL) demonstrate an upregulation of aggrecan gene expression with an induction of 2.7-fold. In the presence of IL-1 or LPS, however, aggrecan gene expression is suppressed by ~50% and 70%, respectively. Importantly, co-incubation with LfcinB significantly reverses IL-1 (Fig. 2E, *lane 4*, $p<0.05$) or LPS (Fig. 2E, *lane 6*, $p<0.05$) mediated suppression of aggrecan expression suggesting that LfcinB exerts its anabolic effects, in part, *via* antagonizing IL-1 and LPS-mediated suppression of aggrecan gene expression in NP cells.

Given the above findings demonstrating a significant LfcinB-mediated increase in pericellular matrix formation in bovine NP cells via particle exclusion assay [Fig. 2A (*d*), (*e*), (*f*)], we further examined whether LfcinB stimulates the expression of genes specific to the pericellular matrix, such as biglycan or decorin. Quantitative PCR results suggest that LfcinB (100 µg/mL) significantly upregulates pericellular matrix-specific components (i.e., biglycan and decorin) in a concentration-dependent manner in bovine NP cells (Figure 2F; $p<0.05$).

LfcinB suppresses IL-1 and LPS-mediated expression and production of catabolic proteases in NP cells

MMPs and ADAMTS family members are key matrix-degrading enzymes in both articular cartilage and the IVD (Bau et al., 2002; Le Maitre et al., 2004; Martel-Pelletier et al., 2001). Accordingly, given that inhibition of matrix-degrading enzyme expression positively regulates cartilage matrix homeostasis (An et al., 2003a; An et al., 2003b), we examined the potential for LfcinB to antagonize IL-1 and LPS-mediated cartilage degrading enzyme production (Gege et al., 2011). Monolayers of bovine NP cells were treated with LfcinB (100 µg/mL), IL-1 (10 ng/mL), LPS (10 µg/mL) and LfcinB plus IL-1 or LPS for 24 hours (Fig. 3), followed by real-time qPCR for mRNA and western blotting analyses for protein level of the following cartilage degrading enzymes: MMP-1, MMP-13, MMP-3, ADAMTS-4, and ADAMTS-5 (Fig. 3A–E, respectively). The treatment of cells with LfcinB alone significantly inhibits multiple MMPs [MMP-1 (Fig. 3A; $p<0.01$), MMP-13 (Fig. 3B; $p<0.05$)] and ADAMTS-5 (Fig. 3E; $p<0.01$) at mRNA levels. No significant baseline change was observed for MMP-3 (Fig. 3C) or ADAMTS-4 (Fig. 3D). As expected, IL-1 and LPS markedly upregulate matrix-degrading enzyme expression compared to controls. Importantly, after co-incubation with LfcinB, these catabolic effects mediated by IL-1 and LPS are significantly attenuated represented by suppressed cartilage degrading enzyme expression at both mRNA and protein levels ($p<0.01$ for MMP-1, MMP-13, MMP-3, and ADAMTS-5; $p<0.001$ for ADAMTS-4). While LfcinB has a minimal effect on the mRNA level of ADAMTS-4 after stimulation of cells with IL-1, it has a striking anti-catabolic effect on LPS-stimulated ADAMTS-4 at both mRNA and protein levels. These results demonstrate that the treatment of LfcinB exerts potent anti-catabolic activity in disc by antagonizing IL-1 and LPS.

LfcinB inhibits IL-1 and LPS-induced catabolic protease activity in both bovine and human NP cells

Given that LfcinB effectively suppresses multiple cartilage degrading enzymes that are upregulated by both IL-1 and LPS, we further analyzed the role of LfcinB in antagonizing IL-1 and LPS-induced activities of catalytic enzymes. Bovine NP cells cultured in monolayer were stimulated with IL-1 (10 ng/mL) or LPS (10 µg/mL) in the presence or absence of LfcinB (100 µg/mL), followed by zymographic analyses. Results reveal that stimulation of NP cells with IL-1 and LPS strikingly enhance catalytic activity, represented by hydrolyzed clear bands of gelatin gel, compared with the levels detected in control (Fig. 4A; $p<0.01$), and this stimulation of enzyme activity was significantly inhibited in the presence of LfcinB in bovine NP cells.

Previously, we reported that IL-1 stimulates MMP-13 expression that is correlated with catalytic activity, as reflected by the digested clear zone on zymography in human disc NP cells (Ellman et al., 2011). To ensure that the inhibition of catabolic enzyme activity by LfcinB is also effective in human disc tissues, we assessed specific MMP-13 enzyme activity by ELISA that detects the activated form of MMP-13 (Fig. 4B). The co-incubation of cells with LfcinB and IL-1 or LPS results in a significant attenuation of both IL-1 (Fig 4B, *lanes 3–4*, $p<0.01$) and LPS-induced MMP-13 enzyme activity (Fig 4B, *lanes 5–6*, $p<0.01$), demonstrating that LfcinB effectively antagonizes MMP-13 enzyme activity that is stimulated by IL-1 and LPS in human discs.

LfcinB attenuates the catabolic factor-mediated stimulation of pro-inflammatory and oxidative stress-associated genes

Both nitric oxide (NO) and IL-6 are known to play an important role in cartilage metabolism, most notably cartilage breakdown (Brenn et al., 2007; Burke et al., 2002a; Castro et al., 2006). Further, increased expression of nitric oxide synthase, a gene responsible for the production of NO, has been associated with cartilage destruction in both knee joints (Pelletier et al., 1999) and spine discs (Kohyama et al., 2000). Importantly, a reduction of inducible nitric oxide synthase (Kokkinos et al.) is closely linked to a reduction in tissue levels of multiple MMPs (Pelletier et al., 1999). We therefore examined the potential for LfcinB to attenuate these catabolic molecules in bovine NP cells. Stimulation with IL-1 and LPS dramatically induce iNOS at the level of mRNA reflected by a 10-fold and 15-fold induction, respectively, compared to control (untreated; assigned as “1”; Fig. 5A). Co-treatment with LfcinB significantly suppresses IL-1-stimulated iNOS expression ($p<0.01$) compared to treatment with IL-1 alone (Fig. 5A, *lanes 3–4*), with a similar but less dramatic inhibition noted after stimulation with LPS and LfcinB (*lanes 5–6*). Treatment of NP cells with IL-1 and LPS upregulates IL-6 at the level of mRNA by a fold-induction factor of 6.5 and 5.5 compared to control, respectively (Fig. 5B, *lanes 3 & 5*; $p<0.01$), and after co-treatment with LfcinB, IL-6 gene induction is suppressed by approximately 50% (IL-1-treated cells) and 60% (LPS-treated cells), compared to treatment with each factor alone ($p<0.01$).

Similar patterns were also found for the toll-like receptor (TLR)-2 and TLR-4 (Fig. 5C & 5D). TLR-2 and TLR-4 have both been theorized to play an important regulatory role in inflammatory responses within articular cartilage, and are highly upregulated by catabolic cytokines (Campo et al., 2011; Kim et al., 2006; Papathanasiou et al., 2011). Treatment of bovine NP cells with IL-1 leads to >2-fold induction of TLR-2 (Fig. 5C, *lane 3*; $p<0.01$) and >3-fold induction of TLR-4 (Fig. 5D, *lane 3* $p<0.01$), and pretreatment of these cells with LfcinB abolishes IL-1-induced stimulation of both receptors, returning back to near control levels (Fig. 5C & D, *lane 4*). Similar results are found after treatment with LPS, as LfcinB mediates a significant downregulation of LPS-induced stimulation of both TLR-2 and TLR-4, with a more potent impact of LfcinB on TLR-2 than TLR-4 (Fig. 5C & D, *lanes 5–6*, $p<0.01$). Taken together, our results reveal the potential for LfcinB to induce anti-oxidative and anti-inflammatory pathways in discs, specifically *via* the inhibition of the TLR-2 and -4 pathways.

LfcinB antagonizes IL-1 and LPS-induced PG depletion in rabbit and mouse disc organ culture

Given the *in vitro* results obtained above, we sought to elucidate potential physiologic effects of LfcinB in an *ex vivo* organ culture model. Both rabbit and mouse discs were intradiscally injected *en bloc* (Fig. 1) with LfcinB [200 µg for rabbit (Fig. 6A), 100 µg for mouse per disc (Fig. 6B)], dissected and subsequently stimulated with IL-1 (100 ng/mL) or LPS (10 µg/mL) in culture media. After 14 days of organ culture, incubation of rabbit

lumbar discs in the presence of IL-1 significantly suppresses PG content [Fig. 6A (b)] compared to intact control [Fig. 6A (a)]. By contrast, treatment with LfcinB by intradiscal microinjection significantly attenuates IL-1-induced PG loss, and maintains the integrity of the disc matrix structural components [Fig. 6A (c)]. Further, there are visible changes in the cellularity within the ECM in the presence of IL-1 [Fig. 6A (e)] compared to control [Fig. 6A (d)]. These changes, reflecting disintegration within the ECM, are significantly attenuated in the presence of intradiscal microinjection of LfcinB (f) without significant alterations in cell viability assessed by Live/Death assay of the disc cells by fluorescent microscopy examination [Fig. 6A (g-j); $p > 0.1$]. Our data suggest that the cell survival rate after 14 days of organ culture period for rabbit IVDs are greater than 90%, reflecting that disc organ culture conditions have no significant effect on IVD cell survival.

Likewise, as demonstrated in a mouse *ex vivo* organ culture model [Figure 6B ((a)-(c))], the presence of IL-1 induces severe depletion of PG [Fig. 6B (b)] compared to intact control [Fig 6B. (a)] after 14 days, while intradiscal microinjection of LfcinB significantly attenuates PG loss induced by IL-1 [Fig. 6B (c)]. Similar results are found in mice discs after LPS injection (10 $\mu\text{g/mL}$). Injection of LPS [Fig. 6B (f)] depletes PG in mice discs compared to control [Fig. 6B (e)], and intradiscal microinjection of LfcinB attenuates PG loss induced by LPS [Fig. 6B (g)], revealing a significant potential of LfcinB to induce anti-catabolic effects *ex vivo*.

DISCUSSION

The major new findings from this study reveal the potent anti-inflammatory and anti-catabolic effects of LfcinB on IVD homeostasis *in vitro* and *ex vivo*. Our data demonstrate that intradiscal administration of LfcinB effectively counteracts IL-1 and LPS-induced catabolism in an *ex vivo* IVD culture model, suggesting a potential therapeutic benefit of LfcinB in the treatment of disc degeneration. In the bovine disc, *in vitro* cell culture studies reveal that LfcinB abolishes the IL-1 and LPS-mediated suppression of PG accumulation, resulting in an upregulation of PG synthesis, aggrecan expression, and pericellular matrix formation via an upregulation of biglycan and decorin. Simultaneously, LfcinB antagonizes IL-1 and LPS-induced upregulation of various cartilage-degrading enzymes, including MMP-1, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 at both mRNA and protein levels, illustrating a profound anti-catabolic role of LfcinB. Further, it is well-known that IL-1 and LPS markedly induce inflammatory and oxidative mediators such as IL-6, iNOS and TLRs *via* auto- and/or paracrine regulatory mechanisms (Chen et al., 2005; Evans et al., 1996; Garcia-Arnandis et al., 2010; Lotz, 1999; Scher et al., 2007; Studer et al., 1999; Weinberg et al., 2007). Our results are the first to shed light on specific anti-inflammatory and anti-oxidative effects of LfcinB in disc NP tissue *via* the suppression of IL-1 and LPS-stimulated IL-6, TLR-2/4 and iNOS expression.

The ability of LfcinB to antagonize the effects of IL-1 and LPS in this study reveals the significant potential for this molecule to induce powerful anti-inflammatory effects in bovine disc tissue. IL-1 is a well-known inflammatory mediator that induces degeneration in both articular cartilage and IVD tissue (Akyol et al.; Lee et al.; Page et al.; Yu et al., 2009). IL-1 not only stimulates the production of destructive enzymes in cartilage homeostasis, but also drives the expression of multiple pro-inflammatory cytokines and inhibits PG synthesis in human articular chondrocytes and disc cells. Similarly, LPS stimulates the production of destructive enzymes in cartilage homeostasis, drives the expression of multiple pro-inflammatory cytokines, inhibits PG synthesis in human articular chondrocytes (Bobacz et al., 2007) and bovine NP but not AF cells (Aota et al., 2006), induces early inflammatory arthritis with synovitis and bone/cartilage destruction via direct intra-articular injection (5–50 $\mu\text{g/joint}$ LPS) (Akahoshi et al., 1994; Idogawa et al., 1998; Matsukawa et al., 1993), and

exacerbates collagen-induced inflammatory arthritis in mice after oral administration (Yoshino et al., 1999). Recently, Liu and colleagues suggested that the compound icariin may exert protective effects on LPS-induced arthritis in neonatal mice chondrocytes via inhibition of nitric oxide and MMP synthesis (Liu et al., 2010). Here, we show similar results using LfcinB, revealing a potential therapeutic role in disc degeneration. It should also be noted, however, that IL-1 attenuates the anabolic effects of LfcinB on PG accumulation (Fig. 2A), suggesting that while LfcinB may antagonize the activity of IL-1, IL-1 may also have a negative effect on LfcinB, retaining at least some of its catabolic/degradative properties in bovine NP cells, even in the presence of LfcinB.

Our long-term alginate bead culture experiments reveal that LfcinB is not only anti-catabolic but also serves an important anabolic role in bovine disc tissue *via* increases in PG deposition and synthesis of the pericellular matrix. This is demonstrated in both qualitative (particle exclusion assay; Fig. 2A) and quantitative (qPCR; Fig. 2E,F) experiments. While stimulation of aggrecan expression by LfcinB is partly responsible for the increased PG production by LfcinB in disc NP cells, the LfcinB-mediated upregulation of well-known pericellular components biglycan and decorin reveal the therapeutic potential for LfcinB to induce substantive increases in matrix formation. Further, given that the overproduction of MMPs and aggrecanases are closely associated with degenerative states in discs (Martel-Pelletier et al., 2001), the LfcinB-mediated suppression of IL-1 and LPS-stimulated MMP or aggrecanase expression may be, at least in part, responsible for the increased matrix formation visualized in disc cells after stimulation with LfcinB. We are the first to reveal the potent anti-catabolic effect of LfcinB *via* antagonism of LPS-mediated activity in disc tissue.

Another novel finding from this study is the anti-inflammatory potential of LfcinB *via* suppression of TLR-2 and TLR-4. TLRs are actively involved in both innate and adaptive immune responses, and TLR signaling pathways are known to increase pro-inflammatory cytokines, TNF- α , IL-1, IL-6, and IL-8, thus mediating inflammation and pain pathways (Janeway and Medzhitov, 2002; O'Neill and Dinarello, 2000). In particular, TLR-2 and TLR-4 are highly upregulated in cartilage with advanced OA (Kim et al., 2006). Overexpression of TLR-2 is observed in degenerative human disc tissue components, including discs, facet joints and facet joint capsular tissues, compared to those in age-matched normal tissues (unpublished data). In addition, it has been reported that, upon stimulation of human OA cartilage with IL-1, TNF- α , peptidoglycan or LPS, chondrocytes significantly upregulate the expression of TLR-2, suggesting a potential critical role of TLR-2 in cartilage degeneration (Kim et al., 2006).

In addition to TLR-2, TLR-4 may also play a critical role in cartilage degradation. Bobacz and colleagues recently demonstrated an LPS-mediated decrease in PG synthesis, aggrecan production, and type II collagen expression in an *in vitro* model using murine and human articular chondrocytes via signaling through TLR-4 (Bobacz et al., 2007). The activation of TLR-4 by LPS also stimulated an upregulation of IL-1, and these results were antagonized by bone morphogenetic protein-7 (BMP-7) and IL-1 receptor antagonist (IL-1Ra) (Bobacz et al., 2007). The results of this study demonstrate that LfcinB, much like BMP-7 and IL-1Ra, is indeed capable of slowing IL-1- or LPS-mediated induction of TLR-2 and TLR-4, with more potent effects on the expression of TLR-4, suggesting that this pathway may be of particular importance in enabling the profound anti-catabolic activity of LfcinB on disc cells. Further studies are indeed warranted to more clearly elucidate the specific downstream signaling cascades mediated by catabolic factors with and without LfcinB on TLR-2 and TLR-4 expression.

In our previous study and those from other groups, resveratrol, a natural antioxidant isolated from red wine, was shown to have anti-oxidative and anti-catabolic effects in articular

cartilage (Elmali et al., 2007; Elmali et al., 2005) and bovine IVD tissue (Li et al., 2008b). The findings from this study reveal a unique similarity between LfcinB and resveratrol. Both factors exert anabolic, anti-catabolic, anti-oxidative, and anti-inflammatory effects in bovine NP tissue. LfcinB may also play a role in pain pathways *via* its effects on NOS and IL-6, which has yet to be studied in the literature pertaining to resveratrol.

It has been reported that the bovine form of Lfcin, LfcinB, has the strongest biological activity compared with Lfcin derived from other species such as human, murine and caprine origin (Vorland et al., 1998). The biological effects of LfcinB against IL-1 and LPS-induced cartilage degradation appear to be common and generalizable between different species. Our *ex vivo* organ culture results reveal that LfcinB generates consistent biological outcomes using disc tissues obtained from both rabbit and mouse sources in addition to bovine tissue. Further, we demonstrate anti-catabolic activity in LfcinB in human tissues via use of gelatin zymography (Fig. 5). *In vivo* animal studies using mice (Guillen et al., 2000) and rat (Hayashida et al., 2004) joints demonstrate anti-inflammatory, anti-catabolic and anti-analgesic properties of human lactoferrin and bovine lactoferrin injections, respectively, suggesting possible preventative and therapeutic effects of lactoferrin on articular cartilage degeneration. LfcinB has also been shown to block FGF-2- and IL-1-mediated catabolic effects in endothelial cells (Mader et al., 2006), suggesting that an anti-catabolic role of LfcinB may be generalizable between different species and tissues.

There are several recognized limitations of this study that must be taken into account. First, we have addressed the role of LfcinB in normal discs from young adult bovine animals in the presence and absence of IL-1 and LPS. However, the biological role of LfcinB under degenerative or regenerative conditions remains unknown as incubation with IL-1 and LPS does not represent actual degenerative conditions. Future studies are warranted to address its effects using animal models of disc or facet joint degeneration (Ellman et al., 2011; Kim et al., 2011b; Kim et al., 2011c). Next, while our results demonstrate that LfcinB significantly antagonizes catabolic factor-mediated induction of MMP-13 enzyme activity in human NP cells, further evaluation using human tissues [NP and annulus fibrosis tissue (AF)] may generalize beneficial effects of LfcinB to the human spine. Third, the current study reports the suppressed catabolic activity of IL-1 and LPS by LfcinB and begins to elucidate the complex downstream effects involved in cartilage homeostasis via TLR-2 and -4 signaling, but a detailed analysis of the specific cell signaling pathways and molecular mechanisms underlying these findings remain largely unknown, and are outside the scope of this paper. Further studies are required to explore the precise downstream mechanisms and molecular crosstalk within the signaling networks induced by LfcinB to influence IVD homeostasis. Finally, the findings reported here relate only to *in vitro* and *ex vivo* culture models, which fails to adequately represent the complex variety of factors that may influence degenerative disc disease *in vivo*. While LfcinB is relatively stable and able to withstand wide ranges in pH, particularly on ingestion (Kuwata et al., 1998), *in vivo* studies are necessary to optimize the dose and administration route of LfcinB. Subsequent animal studies will allow us to elucidate the potential for LfcinB to be used as therapy for disc regeneration and anti-nociceptive treatments in animal models of disc degeneration in the future.

CONCLUSION

LfcinB exerts potent anti-inflammatory, anti-catabolic, and anti-oxidative effects in bovine, mouse, and rabbit disc cells *in vitro* and *ex vivo* via an antagonistic biological effect against IL-1 and LPS-mediated cellular activity. LfcinB may play an important role in the future treatment and prevention of disc degeneration, and its potent anti-IL-1 and anti-LPS activity elucidates a possible role of this peptide in tissue engineering in the future.

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Figure 1. Intradiscal Injection of rabbit lumbar discs

The IVDs of rabbits were dissected under sterile conditions within 12 hours after sacrifice for *ex vivo* organ culture model. *En bloc* intradiscal injections of LfcinB (200 μ g per disc) were performed in rabbit lumbar spine discs using a 26-gauge needle (50 μ L volume) (A), followed by isolation and dissection of each disc using an electronic microsaw (Stryker Biotech) (B).

A. Particle exclusion assay

C. Cell Viability Assay

10 µg/mL Lfcin B	-	+	-	+	-	+
1 ng/mL IL-1	-	-	+	+	-	-
1 µg/mL LPS	-	-	-	-	+	+

D. ^{35}S incorporation assay

E. Real-time qPCR

F. Real-time qPCR

Figure 2. LfcinB counteracts the catabolic effects of pro-inflammatory mediators IL-1 and LPS on PG accumulation in CM of NP cells

NP cells in alginate beads were treated with LfcinB (10 $\mu\text{g/ml}$), IL-1 (1 ng/ml), LPS (1 $\mu\text{g/ml}$), or LfcinB plus IL-1/LPS for 21 days. (A) NP cell pericellular matrix accumulation after 21-day culture was visualized using exclusion assay. A representative cell in each treatment group was photographed using an inverted phase-contrast microscope. The CM is defined as the area between the excluded erythrocytes and NP cell plasma membrane. (B) At the end of the 21-day culture period, the amount of PG in the CM was measured by DMMB assay, and normalized by DNA content. (C) Cell survival was measured by harvesting cells from alginate beads using Calcein AM to stain live cells (green) and ethidium bromide homodimer 1 to stain dead cells (red). Survival assay was conducted weekly (7, 14, and 21 days of culture) by counting at least 100 cells in triplicate for each data point. (D) NP cells were subjected to indicated treatment conditions for 7 days. At the end of the culture period, PG synthesis was measured during the last 4 hours of culture using ^{35}S -sulfate incorporation, and was normalized by DNA content. (E, F) Monolayer NP cells were treated with LfcinB (100 $\mu\text{g/ml}$), IL-1 (10 ng/ml), LPS (10 $\mu\text{g/ml}$) or LfcinB plus IL-1/LPS for 24 hours in serum-free medium. Total RNA was extracted for real-time qPCR analyses of aggrecan (2E) or biglycan and decorin (2F) induction. Gene expression was normalized by individual β -actin expression, and expressed as mean of fold-change $\pm$ S.D. (standard deviation) compared with control level. A value of $p < 0.05$ indicates a significant difference in ANOVA.

A. MMP-1

B. MMP-13

C. MMP-3

100 μ g/mL Lfcin B	-	+	-	+	-	+
10 ng/mL IL-1	-	-	+	+	-	-
10 μ g/mL LPS	-	-	-	-	+	+

D. ADAMTS-4 **E. ADAMTS-5**

Figure 3. LfcinB downregulates IL-1 and LPS-induced production of catabolic proteases in NP cells

Bovine NP cells in monolayer were cultured with LfcinB (100 µg/mL), IL-1 (10 ng/mL), LPS (10 µg/mL) or LfcinB plus IL-1/LPS for 24 hours in serum-free medium. Total RNA was extracted for real-time qPCR analyses of (A) MMP-1, (B) MMP-13, (C) MMP-3, (D) ADAMTS-4, and (E) ADAMTS-5 gene expression. Level of gene expression was normalized by individual β -actin expression, and expressed as mean of foldchange $\pm$ S.D. (standard deviation) compared with control level. A value of $p < 0.05$ and $p < 0.01$ indicate a significant and a highly significant difference in ANOVA, respectively. To assess LfcinB-mediated catabolic protease repression at the level of protein, conditioned medium were collected and subjected to western blotting.

Figure 4. Inhibition of LfcinB on IL-1 and LPS-activated catalytic enzyme activity

Bovine NP cells were treated with IL-1 (10 ng/mL) or LPS (10 µg/mL) in the presence or absence of LfcinB (100 µg/mL) for 24 hours followed by assessments of enzyme activity using (A) zymography and (B) MMP-13 activity ELISA. Gelatin zymography was performed by loading equal volumes of the conditioned media sample on polyacrylamide gel. Band images were digitally captured and intensity of bands (pixels/band) was obtained using the ImageJ densitometry analysis software in arbitrary optical density units. MMP-13 activity was assessed by Active MMP-13 ELISA using a highly specific monoclonal antibody for the activated form of human MMP-13 (sensitivity, 7 pg/mL) in triplicates and human NP cells in monolayer (n=3). Data shown are cumulative of three experiments. OP values presented as mean $\pm$ standard deviation; data without a common letter differ, $p < 0.01$.

Figure 5. LfcinB downregulates inflammation- and oxidative stress-related gene expression in bovine NP cells

NP cells in monolayer were treated with LfcinB (100 μg/mL), IL-1 (10 ng/mL), LPS (10 μg/mL), or LfcinB plus IL-1 or LPS for 24 hours in serum-free medium. Total RNA was extracted for real-time qPCR analyses targeting expression of (A) iNOS, (B) IL-6, (C) TLR-2, and (D) TLR-4. Gene expression was normalized by individual β-actin, and expressed as mean of fold-change ± S.D. compared with control level. A value of $p < 0.01$ indicates a highly significant difference between groups in ANOVA.

A. Rabbit disc *ex vivo* organ culture

B. Mouse *ex vivo* organ culture

Figure 6. Histological assessments and cell viability (original magnification: X40)

Lumbar spine discs of rabbit [A] or mouse [B] were dissected after *en bloc* intradiscal microinjection with LfcinB at the concentration of either 100 μ g for mouse or 200 μ g for rabbit per disc using a 26-G (50 μ L in volume) or 30-G needle (1.5 μ L in volume), respectively. Injected discs were then separated and maintained for 14 days in DMEM/Ham's F-12 medium supplemented with 1% mini-ITS in the presence or absence of catabolic cytokine IL-1 (100 ng/mL). Harvested discs were fixed in 4 % paraformaldehyde, and then decalcified in EDTA followed by paraffin embedding. Serial disc sections of exactly 5- μ m thickness were prepared for slides, and Safranin O-fast green staining was performed [A (a)-(f) for rabbit; B (a)-(c) for mouse, each treatment n=3]. [A (g)-(i)] After 14 days of *ex vivo* organ culture, rabbit lumbar disc cell viability was assessed by fluorescent microscopy using the LIVE/DEAD staining kit. Sample disc tissue was dissected and enzymatically digested followed by incubation in 10 μ M calcein AM green and 1 μ M ethidium homodimer-1 for 30 min followed by visualization. [A (j)] The % cell viability was calculated by counting at least 100 cells in triplicate for each treatment (live/dead cell counts, n=3 for each treatment).

Table 1

Gene	Primer Seq.	NCBI Gene No.	Annealing T _m (°C)
Aggrecan	Forward: 5'-TGAAACCACCTCCACCTTCCATGA-3' Reverse: 5'-TCAAAGGCAGTGGTTGACTCTCCA-3'	NM_173981.2	55
TIMP-3	Forward: 5'-ACGCGTTCTGCAACTCAGACAT-3' Reverse: 5'-TGGTGTAGACCATCGTGCCAAA-3'	NM_174473.3	55
TLR-2	Forward: 5'-TAAGGATGCCTGGCCCTTCCTTCAA-3' Reverse: 5'-GGCCACTGACAAGTTTCAGGCATT-3'	NM_174197.2	55
TLR-4	Forward: 5'-GCGTACTTGGACAAATTCTCAGGG-3' Reverse: 5'-GAGAGAACTGAGCTTCAATGCAGG-3'	NM_174198.6	55
IL-6	Forward: 5'-TCCAATCTGGGTTCATCAGGCGA-3' Reverse: 5'-TTCCCTCATACTCGTTCTGGAGGT-3'	NM_173923.2	55
MMP-13	Forward: 5'-ACCCTTCCTTATCCCTTGATGCCA-3' Reverse: 5'-AAACAGCTCTGCTTCAACCTGCTG-3'	NM_174389.2	55
MMP-3	Forward: 5'-GTTCCGCCTTTCTCAGGATGATGT-3' Reverse: 5'-ATCGAAGGACAAGGCAGGATCACA-3'	AF135232	55
MMP-1	Forward: 5'-ATGTCACACCCTTGACCTTCACCA-3' Reverse: 5'-TTCCACCAGGTCCATCAAAGGGA-3'	NM_174112.1	55
ADAMTS-4	Forward: 5'-AGTTCGACAAGTGCATGGTGTGTG-3' Reverse: 5'-TGGTGACCACGTGTTGTATCCGT-3'	NM_181667.1	55
iNOS	Forward: 5'-TGACATTGACCAGAAGTTGTCCCAGC-3' Reverse: 5'-TTGCCGCTGACATCGAATGTCTCA-3'	NM_001076799.1	60
b-actin	Forward: 5'-AAGAGATCAATGACCTGGCACCCA-3' Reverse: 5'-ACTCCTGCTTGTGTATCCACATCT-3'	BT030480.1	55