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The Action of Resveratrol, a Phytoestrogen Found in Grapes, on the Intervertebral Disc

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Abstract

Study Design—Basic science, biologic study.

Objective—To determine the potential benefits of using resveratrol (RSV) for intervertebral disc (IVD) matrix repair and regeneration.

Summary of Background Data—The phytoestrogen RSV is a natural compound found in various plants including grapes and red wines. RSV has been reported to provide a protective effect on articular cartilage in rabbit models for arthritis, but its effect on spine cartilage is unknown.

Methods—We studied the effect of RSV on bovine IVD cartilage homeostasis by assessing MMP-13 (potent catabolic factor) production, proteoglycan (PG) accumulation and synthesis, and the interaction between RSV and known catabolic factors such as bFGF or IL-1. To understand the molecular mechanisms by which RSV modulates MMP-13 and PG production, we also investigated its downstream target regulatory molecules.

Results—Stimulation of bovine disc cells cultured in monolayer with bFGF or IL-1 augmented the production of MMP-13 and ADAMTS-4 at the transcriptional level and this augmentation was blocked by RSV. Incubation of nucleus pulposus cells with RSV for 21 days significantly increased PG accumulation per cell in a dose-dependent manner, increased PG synthesis, rescued PG losses induced by catabolic reagents bFGF and IL-1, and promoted cell survival to levels seen after incubation with the anabolic protein BMP7 100 ng/mL. Protein-DNA interaction array results suggest that RSV effectively suppresses downstream target molecules of bFGF and IL-1 responsible for oxidative stress, proliferation, and apoptosis.

Conclusion—Resveratrol is a potent anabolic mediator of bovine IVD cartilage homeostasis, revealing its potential as a unique biologic treatment to slow the progression of IVD degeneration. These data suggests RSV may have considerable promise in the treatment of disc degeneration.

Keywords

resveratrol; intervertebral disc degeneration; regeneration; MMP-13; anabolic

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The manuscript submitted does not contain information about medical device(s)/drug(s).

The lifetime prevalence of back pain in the United States is 70% to 85% with roughly 10% to 20% of the population experiencing chronic symptoms.¹ Although the etiology of back pain is likely multifactorial, it has been associated with intervertebral disc (IVD) degeneration.^{2,3} The pathogenesis of degenerative disc disease is thought to be induced mechanically and mediated biologically, often concurrent with aging changes.⁴ Current treatments for low back pain are mainly symptomatic or involve surgical procedures that are destructive to the IVD. Most of these treatment strategies target symptomatic relief but make no attempt to interfere with early biochemical and pathophysiologic processes involved in degeneration. As an alternative to the surgical repair or removal of a diseased disc, biologic treatments capable of promoting IVD repair and restoring physiologic function have been considered, and clinical trials for spine and joint cartilage repair are underway.⁵⁻⁷

The IVD consists of tough outer rings, termed collectively the annulus fibrosus (AF), and a gelatinous inner core, the nucleus pulposus (NP). This unique structure has both shock absorbing properties and the ability to resist deformation on mechanical loading. The AF is composed mainly of collagen, whereas the NP is largely composed of proteoglycans (PGs), principally aggrecan. It has been suggested that the degenerative process begins in the NP and is associated with progressive loss of PGs.⁸

Disc cells residing in the AF and NP regulate homeostasis through metabolic activities that are modulated by a variety of stimuli, including cytokines and growth factors acting in a paracrine and/or autocrine fashion. The cells in the normal disc of human adults maintain the matrix in which they reside at a steady state. Degeneration of the IVD may result from an imbalance between the anabolic and catabolic processes and loss of this steady state metabolism.⁹ IVD damage caused by mechanical injury, inflammation, or aging may change the structure of the IVD, and cause loss of matrix homeostasis by promoting catabolic pathways and/or suppressing anabolic responses. Pro-inflammatory cytokines and growth factors such as interleukin-1 (IL-1)¹⁰⁻¹² and basic fibroblast growth factor (bFGF)¹³ have been implicated in degenerative disc disease.

The catabolic involvement of matrix metalloproteases (MMPs), specifically the pathogenic role of MMP-13 (collagenase-3), has already been demonstrated in the degeneration of the IVD.¹² In addition, MMP-13 is highly expressed in several other pathologic contexts, including osteoarthritis,¹⁴ rheumatoid arthritis,¹⁵ and invasive cancer.¹⁶ It is clear that in both articular and IVD cartilage, the cells are responsible for the destruction of their own matrix *via* the release of destructive enzymes including MMP-13 and ADAMTS-4, a well-known aggrecanase.¹⁷ In the IVD, both MMP-13 and ADAMTS-4 act by breaking down aggrecan, the most abundant PG, leading to NP destruction and further disc degeneration.^{8,17,18}

The phytoestrogen resveratrol (trans-3,4',5-tri-hydroxystilbene; RSV) is a natural polyphenol compound found in various plants including grapes and red wines. The anti-inflammatory, antioxidant, cardioprotective, and antitumor properties of RSV have already been well-documented.¹⁹⁻²⁷ RSV is believed to be one of the compounds responsible for the health benefits of moderate red wine consumption.^{28,29} More recently, RSV has been reported to provide a protective effect on articular cartilage in rabbit models of OA and RA.^{30,31} Using these models, Elmali *et al* (2005) demonstrated that injections of RSV significantly decreased loss of PG and cartilage destruction in arthritic rabbit knees. However, it is not known if RSV exerts similar protective effects on degenerating IVDs.

The aim of the present study is to determine the potential benefits of using RSV to retard the progression of IVD degeneration. Specifically, we studied the effect of RSV on IVD cartilage homeostasis by assessing MMP-13 production, PG accumulation, and PG synthesis in the bovine spine IVD, as well as evaluating whether RSV counteracts known catabolic factors

such as bFGF or IL-1. To understand the molecular mechanisms by which RSV modulates MMP-13 and PG production, we also investigated the downstream target regulatory molecules of RSV.

Materials and Methods

IVD Cell Isolation and Culture

Tails from young adult bovine animals (15–18 months old) were purchased from a local slaughterhouse. Coccygeal discs were opened *en bloc*, and the NP and AF portion of each disc were separated. 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 pronase and collagenase P, as previously described.^{32–34} Alginate beads and monolayers were prepared for long-term and short-term studies, respectively. For alginate bead culture, isolated disc cells were resuspended in 1.2% alginate, and beads were formed by dropwise addition into a CaCl_2 solution as previously described.³⁵ Briefly, beads were cultured at 8 beads/well in 24-well plates in 1 mL/well of DMEM/Ham's F-12 medium (1:1) supplemented with 1% mini-ITS+ (insulin-transferrin-selenium).³⁶ Cells were treated with RSV at 10, 50, 100, or 200 $\mu\text{mol/L}$ (Calbiochem, San Diego, CA), bFGF at 10 ng/mL (NCI, Bethesda, MD), and BMP7 at 100 ng/mL (Stryker Biotech, Hopkinton, MA). For all subsequent long-term and short-term experiments, RSV was used at a concentration of 200 $\mu\text{mol/L}$, as this is the maximum concentration found to maintain discs cell viability and provide consistency from experiment to experiment. Culture in the presence of the well-known catabolic cytokine, IL-1 β at 1 ng/mL (Amgen, Thousand Oaks, CA), was used as a control. Triplicate wells were used for each condition. The medium in all cases was changed every other day over a 21-day period before dimethylethylene blue (DMMB) analysis.

For monolayer cultures, isolated NP cells were counted and plated at 8×10^5 cells/cm² as previously described.³⁴ The cells were treated with RSV (200 $\mu\text{mol/L}$), bFGF (10 ng/mL), IL-1 β (1 ng/mL) and IL-1 receptor antagonist (IL-1ra) 100 ng/mL (Calbiochem, San Diego, CA). The supernatant was removed 24 hours after the initiation of each treatment and subjected to immunoblotting, as described below. For nuclear extraction, experiments were terminated 45 minutes after treatment was initiated.

Cell Survival Assay

Survival of cells in alginate was measured using Calcein AM to stain live cells, and ethidium bromide homodimer 1 to stain dead cells following the manufacturer's protocol (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.

Disc Cell Stimulation and Immunoblotting

Experiments were terminated with removal of medium and/or cell lysate preparations as described above. Total protein concentrations of media were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). In each case, an equal amount of protein was resolved by 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane for immunoblot analyses as previously described.^{37,38} Anti-MMP-13 and β -actin antibodies were purchased from R&D System (Minneapolis, MN) and Abcam (Cambridge, MA), respectively, and used for supernatant analysis.

Reverse Transcription and Real-Time Polymerase Chain Reaction

Total cellular RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. Reverse transcription (RT) was carried out with

1- μ g total cellular RNA using ThermoScript RT-PCR system (Invitrogen) for first strand cDNA synthesis in 20 μ L of reaction volume.

For real-time PCR, cDNA was amplified using Bio-Rad MyiQ Real-Time PCR Detection System. RT product was subjected to real-time PCR in a 20- μ L total reaction mixture containing 10 μ L Bio-Rad iQ SYBR Green supermix (Bio-Rad, Hercules, CA). A threshold cycle (C_T value) was obtained from each amplification curve using the iQ5 Optical System Software (Bio-Rad) provided by the manufacturer. Relative mRNA expression was determined using the $\Delta\Delta C_T$ method, as detailed by manufacturer guidelines (Bio-Rad). The housekeeping gene β -actin was used as internal control in the reaction for normalization. The primer sequences and the conditions for their use are summarized in Table 1.

DMMB Assay for Proteoglycan Accumulation and DNA Assay for Cell Numbers

The alginate culture system offers the possibility to quantify the accumulation of matrix components in 2 compartments, the cell-associated matrix (CM) and the further-removed matrix. The CM is in close contact with the cell and is a more sensitive method for analysis of PG accumulation; therefore, PG production in the CM was analyzed. At the end of the 21-day alginate culture period, the medium was removed, and the alginate beads were collected and processed for PG assays using the DMMB binding method, as previously described.³⁶ The CM was separated from the further-removed matrix and PG accumulation per cell was quantified.³⁹ Using PicoGreen (Molecular Probes), cell numbers were determined by assay of total DNA in the cell pellets, as previously described.³³

³⁵S-Sulfate Incorporation Into Newly Synthesized Proteoglycans

The same labeling protocol was used for all cultures. On day 7 of culture in alginate, the 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, the labeling medium was removed and beads were rinsed twice in cold 1.5 mmol/L SO₄ wash medium. Beads were dissolved and digested with 400 μ L papain (20 μ g/mL in 0.1M sodium acetate, 0.05MEDTA, pH 5.53) at 60°C for 16 hours. Sulfate incorporation was measured using the Alcian blue precipitation method, as previously described.⁴⁰ All samples were analyzed in duplicate and normalized for DNA content using Hoechst 33258 as previously described.⁴⁰

Protein-DNA Interaction Array Using Nuclear Extracts

Nuclear extracts were prepared from cells cultured in 6-well monolayer plates using the Nuclear Extraction Kit (Panomics, Inc., Fremont, CA) according to the manufacturer's protocol. The protein concentration was determined using the Micro bicinchoninic acid assay (Pierce, Rockford, IL). Biotin-labeled DNA binding oligonucleotides (TranSignal Probe Mix) were preincubated with nuclear extracts with different treatments to allow for the formation of DNA/protein complexes. The probes in these complexes were then extracted and hybridized to the TranSignal protein/DNA array membranes following the procedure outlined in the Panomics TranSignal spin protein/DNA array kit user's manual. Membrane detection was conducted following the procedure in the Panomics manual.

Statistical Analysis

Analysis of variance was performed using StatView 5.0 software (SAS Institute, Cary, NC). *P* lower than 0.05 were considered significant.

Results

Inhibition of MMP-13 by Resveratrol After Stimulation With Catabolic Reagents

Stimulation of bovine NP and AF cells cultured in monolayer with bFGF (10 ng/mL) or IL-1 (10 ng/mL) augmented the production of MMP-13 at the protein level, and this effect was greater in AF cells than in NP cells (Figure 1A). A greater effect was seen after stimulation with IL-1 than with bFGF. This augmentation of MMP-13 was blocked by RSV 200 $\mu\text{mol/L}$ in both NP and AF cells. When given with IL-1, IL-1 receptor antagonist (IL-1ra) completely abolished MMP-13 protein expression in NP and AF cells. When given with bFGF, however, IL-1ra reduced, but did not abolish, MMP-13 protein expression compared with treatment with bFGF alone, suggesting an inhibitory effect of IL-1ra on bFGF-mediated MMP-13 stimulation.

In addition, real-time PCR results demonstrate that stimulation with bFGF (10 ng/mL; lane 1) caused a 2-fold increase in MMP-13 mRNA expression in NP cells (Figure 1B). However, after addition of RSV 200 $\mu\text{mol/L}$ to the bFGF-stimulated sample, this augmentation of MMP-13 was completely abolished. Treatment with RSV alone (200 $\mu\text{mol/L}$) further decreased MMP-13 transcription to roughly 70% of control. These results show that in the presence of RSV, bFGF-mediated stimulation of MMP-13 was abolished at the transcriptional level and is dependent on *de novo* protein synthesis in bovine NP cells.

Further, real-time PCR results indicate that RSV inhibited the catabolic factor-mediated stimulation of the aggrecanase ADAMTS-4 that has been shown to be associated with NP destruction.¹⁷ Stimulation of bovine NP cells with bFGF (10 ng/mL) increased ADAMTS-4 mRNA by a factor of 1.8 compared with control (Figure 1C). When given with RSV 200 $\mu\text{mol/L}$, this bFGF-induced augmentation of ADAMTS-4 was abolished and reversed, as the relative expression of mRNA decreased by 80% compared with control.

Anabolic Effect of Resveratrol on PG Accumulation in the CM of Bovine NP Cells

Bovine NP cells were cultured in alginate beads for 21 days in the presence of RSV and analyzed using DMMB assay for accumulation of PG in the CM. Incubation of cells with RSV significantly increased PG accumulation per cell in a dose-dependent manner (Figure 2A). At concentrations of 10 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ RSV, PG accumulation increased by 20% and 80%, respectively, compared with control. Interestingly, when cells were treated with RSV at a concentration of 200 $\mu\text{mol/L}$, PG accumulation was slightly greater than that seen in cells incubated with BMP7 (100 ng/mL), a well-known anabolic control, suggesting that RSV has a potent anabolic impact on NP cells.

Further studies demonstrated that RSV rescues PG losses induced by the catabolic reagents bFGF and IL-1 in bovine NP cells (Figure 2B). When given alone, bFGF (10 ng/mL) and IL-1 (1 ng/mL) decreased PG accumulation by 45% and 40%, respectively, compared with control. However, when preincubated with RSV (200 $\mu\text{mol/L}$), PG accumulation markedly increased to almost twice the level of control for both bFGF and IL-1. Treatment with RSV (200 $\mu\text{mol/L}$) alone led to the greatest increase in PG production, increasing PG accumulation to 1.9 times that of control.

Resveratrol-Mediated Increase in PG Accumulation is, in Part, Because of Increased PG Synthesis

To determine if the increase in PG accumulation was mediated by a RSV-induced stimulation of PG synthesis, the incorporation of ³⁵S-sulfate by bovine NP cells into PGs was quantified. The results showed that the stimulation of PG accumulation induced by RSV is at least partly because of increased PG synthesis (Figure 3). When NP cells were incubated with RSV at a concentration of 200 $\mu\text{mol/L}$, PG synthesis increased to approximately 1.8 times that of control,

with levels expressed per μg of DNA. Further, the catabolic factor-mediated suppression of PG synthesis was completely recovered and even reversed in the presence of RSV. Treatment with IL-1 and bFGF, 2 well-known catabolic mediators of PG synthesis, suppressed PG synthesis by 45% and 40%, respectively, compared with control. However, when these catabolic reagents were preincubated with RSV, the decrease in PG synthesis was reversed and synthesis increased to approximately 170% of control for both reagents.

Resveratrol Inhibits Multiple Downstream Regulatory Molecules That Are Activated by bFGF and IL-1

Using nuclear extracts from bovine NP cells, we assessed a multiplex protein-DNA interaction array. The array panel contains a selected set of downstream transcription factors (AP-1 and AP-2, CREB, Ets1/PEA3, E2F1, estrogen RE, NF κ B, Sp1) and signal transducers and activators of transcription (STATs, including STAT3, 4, and 5) that are involved in bFGF- and IL-1-mediated catabolic activity. They are activated after stimulation with bFGF (Figure 4A) and IL-1 (Figure 4B). At baseline levels (control), only Ets1/PEA3 was slightly activated, whereas all other transcription factors were inactive. However, after stimulation with bFGF and IL-1, all transcription factors were activated. In contrast, in the presence of RSV, the bFGF-mediated activation of transcription factors was suppressed and in most cases completely abolished, with the exception of Sp1, which was significantly attenuated after the addition of RSV (Figure 4A). All other transcription factors were rendered inactive. IL-1-mediated activation of transcription factors was also suppressed (AP-1 and AP-2) or completely abolished in the presence of RSV, with the exception of p53. RSV decreased IL-1-mediated activation of p53, a key transcription factor, promoting the apoptotic pathway and suppressing proliferation which was less responsive to RSV treatment (Figure 4B).

Resveratrol Promotes Proliferation and Viability of Bovine NP Cells

When NP cells were incubated for 21 days in alginate with bFGF at 10 ng/mL, the amount of DNA increased by a factor of 7 on day 7 and a factor of 16 on day 21 compared with control, revealing the potent mitogenic capacity of bFGF in bovine NP cells. When NP cells were incubated with 200 $\mu\text{mol/L}$ RSV, the amount of DNA increased by a factor of 1.2 on day 7 and 1.8 on day 21 compared with control, suggesting a slight mitogenic effect of RSV in bovine spine tissue (Figure 5A). However, although stimulation of alginate-cultured NP cells with bFGF decreased cell viability over time (similar to control), treatment with RSV increased and maintained cell viability over time (Figure 5B). For example, on day 21, percentage of live cells in the bFGF-treated group was 87%, compared with 94% in the RSV-treated group, equal to the viability of cells treated with the potent anabolic factor BMP7 (100 ng/mL).

Discussion

This study illustrates the potent anabolic effects of RSV on bovine IVD homeostasis. Treatment with RSV inhibited MMP-13 expression at the transcriptional level, increased PG accumulation, and stimulated PG synthesis in bovine NP cells. In addition, RSV reversed the catabolic effects of bFGF and IL-1 that have been implicated in disc degeneration. Finally, our data shed light on the response of multiple downstream regulatory molecules after stimulation with RSV in the bovine IVD and provide us with a better understanding of the anti-inflammatory, antioxidant, and antiproliferative and anabolic effects of RSV.

Accumulated evidence has indicated that in arthritic cartilage and the degenerating IVD, the overproduction of collagenases, in particular MMP-13, by chondrocytes or disc cells plays a central role in matrix degeneration.^{41–45} Research from our laboratory has previously demonstrated the potent catabolic effects of IL-1 and bFGF in human articular cartilage, particularly *via* the catabolic factor-mediated upregulation of MMP-13.^{34,38} In this study, we

demonstrate the potent upregulation of MMP-13 after treatment with both IL-1 and bFGF in spine tissue. Further, when bovine NP and AF cells were pretreated with RSV, IL-1- and bFGF-mediated stimulation of MMP-13 was completely reversed as MMP-13 protein expression was abolished, and the potent impact of RSV on bFGF-stimulated MMP-13 expression seems to be regulated at the transcriptional level. Also, the bFGF-induced stimulation of ADAMTS-4, a well-known aggrecanase, was reversed by RSV in bovine NP cells (Figure 1C), and RSV antagonized the IL-1-mediated stimulation of ADAMTS-4 gene expression as well (data not shown), further supporting the anticatabolic potential of RSV. These results provide evidence that RSV is indeed capable of slowing some of the catabolic processes involved in disc degeneration.

The anabolic capacity of RSV is demonstrated by our DMMB results. We found that treatment with RSV in alginate culture dose-dependently increased the total accumulation of PG in NP cells and rescued PG losses when combined with bFGF and IL-1. This increase in PG could be because of either decreased PG degradation, increased PG synthesis, or both. Examples of decreased PG degradation include the inhibition of IL-1- or bFGF-stimulated, MMP-13- or ADAMTS-4-mediated destruction of aggrecan, leading to increased accumulation of PG. However, the results of our sulfate incorporation experiments suggest that the increase in PG levels is, at least in part, because of increased PG synthesis as well. After preincubation with RSV, the decrease in PG synthesis seen with IL-1 and bFGF was completely reversed, leading to an anabolic increase in PG synthesis compared with control. Therefore, we propose that RSV increases PG accumulation *via* inhibition of PG degradation and stimulation of PG synthesis.

Our protein-DNA interaction array supports the view that RSV inhibits multiple downstream target molecules of catabolic reagents such as bFGF and IL-1. The target molecules studied are known to be associated with oxidative stress, inflammation, and proliferation,⁴⁶ and nuclear explants from NP cells stimulated with catabolic reagents display these inflammatory markers (Figure 4). Our results suggest that, with the exception of p53, RSV effectively suppresses all downstream target molecules of bFGF and IL-1. Especially, we observed highly activated p53 in the presence of IL-1, suggesting it may be one of the mechanisms by which IL-1 (>1 ng/mL concentration) leads to cell death (data not shown), and RSV improves cell survival (Figure 5B). Taken together, RSV inhibits multiple MAP kinase pathways (specifically the ERK pathway and NF κ B pathway), along with multiple STATs and downstream transcription factors of bFGF and IL-1, illustrating the potential for RSV to exert anabolic, antioxidative, anti-inflammatory, and antiapoptotic effects.

In bovine spine tissues, our results reveal a potent mitogenic effect of bFGF. However, although bFGF increases NP cell proliferation, it fails to increase ECM production in bovine IVD cells, resulting in clustering of cells often seen in degenerative discs.⁴⁷ RSV, on the other hand, only slightly increases cell proliferation and does not form the clusters of cells seen after treatment with bFGF. Additionally, RSV increases NP cell viability in a similar fashion to BMP7. Lee *et al* showed that RSV protects neuroblastoma cells from apoptosis,⁴⁸ whereas Shakibaei *et al* demonstrated the antiapoptotic effects of RSV in human articular chondrocytes.⁴⁹ Together, the increased cell viability seen in bovine NP cells after stimulation with RSV may be because of a reduction of intracellular oxidative stress coupled with inhibition of disc cell apoptosis.

Clinically, several limitations of this study must be taken into account. First, these findings relate only to a cell culture model system, which fails to adequately represent the complex variety of factors that may influence the onset and progression of disc degeneration. Second, increased oral intake of RSV *via* increased red wine or grape consumption may provide patients with systemic benefits *via* antioxidant, cardioprotective, and antitumor effects shown in a variety of tissues.^{19–27} However, given the avascularity of the IVD, oral intake of RSV would

not be a feasible option for treatment of disc degeneration. Rather, direct injections of RSV into the disc may serve as a feasible therapeutic option to slow the progression of degenerative disc disease, and future studies are warranted to elucidate both the potential and concentration of RSV injections to slow disc degeneration *in vivo*. Finally, we have demonstrated that RSV may act to slow the progression of degenerative disease *via* inhibition of NP cell-induced catabolism and stimulation of PG synthesis, but we have not illustrated the potential capacity of RSV to prevent and/or reverse disc degeneration. Therefore, future studies are needed to assess whether the factors initiating disc degeneration are the same as those causing progression, and whether RSV has a direct influence on tissue repair. Nevertheless, the results of this study are the first to reveal the considerable promise of RSV as a unique biologic therapy for treatment of IVD degeneration.

Key Points

- RSV antagonizes catabolic factor-mediated upregulation of matrix-degrading enzymes in bovine disc cells.
- RSV promotes PG synthesis and accumulation in bovine NP cells.
- RSV suppresses downstream target molecules of bFGF and IL-1 responsible for oxidative stress, proliferation, and apoptosis.

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Figure 1.

Inhibition of MMP-13 and ADAMTS4 by resveratrol after stimulation with catabolic reagents. NP and AF cells isolated from bovine IVD were cultured in monolayer in 12-well plates 8×10^5 cells/cm², and were serum starved by changing the media to serum-free DMEM/F-12 with antibiotics for 24 hours before treatment. Cells were then treated with bFGF 10 ng/mL, IL-1 β 1 ng/mL, and RSV 200 μ mol/L, collected after 24 hours, and total RNA was extracted to perform real-time RT-PCR, whereas the conditioned media was subjected to immunoblotting. Results show the IL-1 β and bFGF-induced augmentation of MMP-13 was blocked by RSV in both NP and AF cells. IL-1 β receptor antagonist (IL-1ra) (100 ng/mL) abolishes or reduces MMP-13 protein expression that was increased by IL-1 β or bFGF treatment (**A**). In NP cells,

the bFGF-induced augmentation of MMP-13 and ADAMTS-4 mRNA levels was abolished by RSV 200 $\mu\text{mol/L}$ (**B**, **C**).

Figure 2.

Anabolic effect of resveratrol on PG accumulation in CM of NP cells. NP cells isolated from bovine IVD were cultured for 21 days in 1.2% alginate beads in serum-free medium with mini-ITS+ (insulin-transferrin-selenium; control) or the control medium plus RSV 10 to 200 μ mol/L. Control medium plus BMP7 100 ng/mL was used as a positive control. At the end of the culture period, the beads were dissolved in sodium citrate and cell pellets were separated by centrifugation. The pellet contains cells and CM with PG. The amount of PG in the CM was measured by DMMB assay and normalized to cell numbers using DNA measurement (DMMB/DNA). Samples were measured in triplicate and expressed as a percentage of the day 21 control cultures (mean and SEM). The results show that incubation of cells with RSV 10 to 200 μ mol/L

L significantly increases PG production in a dose-dependent manner (**A**). Further studies demonstrate that RSV rescues PG losses induced by the catabolic reagents bFGF (10 ng/mL) and IL-1 β (1 ng/mL) in bovine NP cells (**B**).

Figure 3.

Resveratrol-mediated increase in PG accumulation is, in part, because of increased PG synthesis. NP cells isolated from bovine IVD were cultured for 7 days in 1.2% alginate in serum-free medium with mini-ITS+ (insulin-transferrin-selenium; control) or the control medium plus RSV 200 μmol/L, IL-β 1 ng/mL, or bFGF 10 ng/mL. PG synthesis was measured during the last 4 hours of culture using [³⁵S]-sulfate incorporation and was normalized to cell numbers by DNA assay. Results are expressed as a percentage of control for triplicate samples (mean and SEM). Data show that PG synthesis increased when NP cells were incubated with RSV, and the catabolic factor-mediated suppression of PG synthesis was completely recovered and even reversed in the presence of RSV.

Figure 4.
Resveratrol inhibits multiple downstream regulatory molecules that are activated by bFGF and IL-1. NP cells isolated from bovine IVD were cultured in 6-well plates, 8×10^5 cells/cm², and serum starved by changing media to serum-free DMEM/F-12 with antibiotics for 24 hours. For treatment, cells were incubated with bFGF 100 ng/mL or IL-1 β 10 ng/mL and/or RSV 200 μ mol/L for 6 hours. Nuclear extracts were prepared from cells. Biotinlabeled DNA binding oligonucleotides (TranSignal Probe Mix) were preincubated with different treatments to allow the formation of DNA/protein complexes. The probes in the complexes were then extracted and hybridized to the TranSignal Protein/DNA array I membranes. These blots contained 54 different transcription factor DNA-binding sites. The response elements on the array are spotted in duplicate: the first row is DNA spotted normally, the second row is DNA diluted 1:10. The right and bottom sides of the array indicate where biotinylated DNA were spotted.

The results show that in the presence of RSV, bFGF-mediated activation of transcription factors known to be associated with oxidative stress, inflammation, and proliferation were significantly reduced (**A**). Similar results were observed in the presence of RSV after stimulation of cells with IL-1 β , except p53 (**B**).

Figure 5.

Resveratrol promotes proliferation and viability of bovine NP cells. NP cells isolated from bovine IVD were cultured for 21 days in 1.2% alginate beads in serum-free medium with mini-ITS+ (insulin -transferrin-selenium; control) or the control medium plus RSV 200 μ mol/L. Cells were treated with BMP7 100 ng/mL as a positive control and bFGF 10 ng/mL as a negative control. Using PicoGreen (Molecular Probes), cell DNA was quantified to represent effects of treatments on cell proliferation (**A**). Cell survival assay was measured using Calcein AM to stain live cells, and ethidium bromide homodimer to stain dead cells. At least 100 cells were counted in triplicate for each data point and percentage of live cells was calculated accordingly (**B**). Result demonstrates that RSV 200 μ mol/L promoted cell survival.

Table 1

Primer Sequence for Real-Time RT-PCR

Gene	Primer Sequences (Forward/Reverse) (5'-3')	Size (Bp)	Annealing Temperature (°C)	Reference Accession No.
MMP13	ACC CTT CCT TAT CCC TTG ATG CCA AAA CAG CTC TGC TTC AAC CTG CTG	110	55°C	NM_174389.2
ADAMTS4	ACT GGG CTA CTA TTA CGT GGA AAA CAC ACA CCA TGC ACT TGTCGA ACT	155	60°C	BC148059.1
β -actin	AAG AGA TCA ATG ACC TGG CAC CCA ACT CCT GCT TGC TGA TCC ACA TCT	141	55°C	BT030480.1