Emodin ameliorates matrix degradation and apoptosis of nucleus pulposus cell and attenuates degeneration of intervertebral disc through LRP1 in vitro and in vivo

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Abstract

Intervertebral disc degeneration (IDD) is closely correlated with Low back pain. Apoptosis and extracellular matrix (ECM) degradation caused by inflammation-mediated NF-κB has been reported to promote IDD. Low-density lipoprotein receptor-related protein-1 (LRP1) was confirmed to negatively regulate NF-κB in many disease. Moreover, emodin has been shown to upregulate LRP1. However, the effect of emodin on IDD, more importantly, the effect of in vivo therapy and mechanism are not clear. This study aimed to investigate the effect of emodin and its target on IDD in vivo and in vitro. Alcian blue staining showed emodin effectively rescued IL-1β-induced ECM secretion. Moreover, the results of western blot showed emodin promoted matrix synthesis, inhibited matrix degradation and apoptosis in NPCs under stimulation of IL-1β. Further study showed emodin inhibited IL-1β-induced NF-κB. In our study, we found emodin increased protein level of LRP1 levels by inhibiting its degradation via the proteasome pathway. In addition, LRP1 knockdown blocked emodin's effects on inhibition of NF-κB, and thus promoted apoptosis and matrix metabolism disorder in NPCs. Emodin effectively alleviated IDD in rat model and siRNA LRP1 injection also reversed beneficial effect of emodin on IDD in rat model. In conclusion, this study showed that emodin has therapeutic effect on IDD by inhibiting matrix metabolism disorder and apoptosis through LRP1 in vitro and in vivo, which was related to LRP1-mediated inhibition of NF-κB. This study provided evidence for emodin as a potential drug for the treatment of IDD.

1. Introduction

Low back pain (LBP) greatly affects people's quality of life and it is a main cause of disability, which brings a huge burden to the social economy [1, 2]. Intervertebral disc degeneration (IDD) accounts for roughly 40% of all cases of LBP[3]. The intervertebral disc (IVD) is an important undertaker to maintain the normal function of the spine[4–6]. Dysfunction of nucleus pulposus cells (NPCs) including extracellular matrix (ECM) degeneration, increased secretion of inflammatory factors, excessive senescence and apoptosis are the main factors leading to IDD[7–9]. Inflammation is a key element leading to apoptosis of NPCs and ECM degradation[7, 10]. Nonsteroidal anti-inflammatory medicines are primarily employed in clinical therapy to alleviate LBP, but it cannot inhibit progression of IDD[11]. Surgical treatment is the last resort, but it is traumatic and has many operation-related complications[12]. Therefore, it is important to find precise drugs targeting NPCs to carry out early intervention.

Recent studies have shown that small molecular drugs have great potential to alleviate IDD[13]. However, the study of small molecular drugs in the treatment of IDD still needs to be further developed, because the signal pathways that regulate cell matrix metabolism and cell fate are still unclear. Emodin is an effective ingredient extracted from the traditional Chinese medicine rhubarb, which has been confirmed to have a variety of pharmacological properties, such as anti-inflammatory[14, 15], antioxidant[16], anti-cancer[17], anti-bacterial[18] effects. In terms of anti-inflammation, researches have demonstrated that emodin is capable of inhibiting the activation of NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammatory bodies induced by lipopolysaccharide (LPS)[19]. In addition, emodin decreased the expression of matrix metalloproteinase 3 (MMP3) and matrix metalloproteinase 13 (MMP13) while
increasing the production of collagen type 2 (COL2) and Aggrecan by inhibiting nuclear factor kappa-B (NF-κB) in interleukin-1 (IL-1β)-treated chondrocytes. Although a recent study has shown emodin can inhibit apoptosis of NPCs by inhibiting reactive oxygen species (ROS) and NF-κB in vitro[20], the effect of emodin on matrix metabolism of NPCs, more importantly, in vivo therapy and its mechanism remain unclear.

Low-density lipoprotein receptor-related protein 1 (LRP1) is a ubiquitous membrane-spanning protein, participating in the endocytosis of many ligands and as a signal receptor to regulate various intracellular signaling pathway. In the study of osteoarthritis, LRP1 not only mediates the phagocytosis and degradation of MMP13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS5) to protect articular cartilage[21], but also inhibits apoptosis in chondrocytes[22]. Upregulation of LRP1 can decrease the production of IL-1β by inhibiting the NF-κB[23]. Furthermore, A study has shown that emodin can upregulate the expression of LRP1. Therefore, we speculate that emodin may regulate LRP1 and thus inhibit NF-κB in NPCs.

In our study, we found emodin inhibited apoptosis and ECM degradation, which was related to LRP1-mediated inhibition of NF-κB. Furthermore, we verified it in vivo. The above has not been studied, and our study fills the gap in literature, which provides strong evidence for emodin to be a potential drug in the treatment of IDD.

2. Methods

2.1. Animals

All animal experiments were conducted under the supervision of Sun Yat-sen University's Institutional Animal Care and Use Committee (IACUC) and approved by the Institutional Research Ethical Committee of Sun Yat-sen University (NO. SYSU-IACUC-2021-000942). All of the experimental animals were 10-week-old male SPF Sprague Dawley (SD) rats (n = 18) obtained from the Laboratory Animal Center of Sun Yat-sen University and divided rats into three groups: sham group (n = 6), IDD group (n = 6), emodin group (n = 6). The operation of the three groups of rats will be illustrated in detail in surgical procedures.

2.2. Surgical Procedures

After fasting for 12 h and water deprivation for 4h, pentobarbital sodium was used to anesthetize rats intraperitoneally (5 mg/100 g body weight). The IDD model was established by annulus fibrosus (AF) needle puncture at the caudal intervertebral disc in rats. Briefly, after disinfecting the skin with 75% ethanol, a 20-gauge puncture needle was used to enter the center of the Co6/7 or Co7/8 intervertebral disc vertically. The puncture depth was 4 mm, and the puncture needle was rotated 360 degrees clockwise and held steady for 30 s. The above methods were used in both the IDD group and the emodin group. The operation in the sham group was similar to that in the other two groups without insertion into the disc. A week after establishing the models, 4 µl emodin (10 µM) was injected into the disc in the emodin group. Considering the influence of the injection volume on the intervertebral disc, 4 µl phosphate-
buffered saline (PBS) was injected into the intervertebral disc in the IDD group. To minimize secondary
dermal damage caused by puncture, a 33-gauge micro-syringe (Hamilton, USA) was used for PBS and emodin
jection. After 4 weeks treatment, the rats were euthanized by an overdose of sodium pentobarbital (150
mg/kg) for magnetic resonance imaging (MRI) and tissue collection. To investigate the effect of LRP1 on
emodin therapy in vivo. After establishing the IDD model and injecting emodin as mentioned above, the
2’ome modified siRNA (2nmol; 4µl) was injected into the rat intervertebral disc 2 times a week for 2
weeks. The intervertebral discs were collected for immunohistochemistry.

2.3. NPC Culture

The rat NPCs utilized in the experiment were immortalized cell lines kindly provided by Dr. Chen Di at the
Department of Orthopedic Surgery, Rush University Medical Center (Chicago, IL, USA)[24]. The
immortalized rat NPC line was created utilizing the ROCK inhibitor Y-27632, which allowed for 50
passages without loss of proliferation and phenotypic characteristics. The immortalized cell line keep the
characteristics of the original cell and can be utilized as a helpful tool for in vitro signal investigations
and drug screening[24]. NPCs were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) with 10%
fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (HyClone, Sv30010, USA) at 37°C in a 5%
carbon dioxide environment. Cell lines were passaged when they reached 80% confluent. No less than
three independent repeated experiments were carried out in in vitro experiments.

2.4. Cell Viability

To explore the toxic effects of different concentrations of emodin on NPCs and the optimum
concentration for follow-up experiments, cell viability was estimated utilizing a Cell Counting Kit-8
(ApexBio, K1018, USA). NPCs were planted into 96-well plates with 2000 cells per well and stimulated
with different concentrations of emodin (Selleckchem, S2295, purity: 98.11%, Houston, TX, USA),
containing 5, 10, 20, 40 and 80 µM, for 24 and 48 h. Optical density (OD) was detected at a wavelength of
450 nm, and drug toxicity was evaluated by the cell viability = [(As-Ab)/(Ac-Ab)] × 100%.

2.5. High Density Culture and Alcian Blue Staining

To examine the ECM secretion capabilities of NPCs, after digestion and centrifugation, approximately
100,000 cells were resuspended in 10 µl medium and planted as micromasses in the center of a 24-well plate.
After 1 hour of cultivation, 1 mL DMEM containing 2% FBS and 1% insulin transferrin selenium
(ITS) was added. NPCs were treated with IL-1β (SinoBiological, 10139-HNAE, China) and different
concentrations of emodin. After 5 days, NPCs were washed with PBS buffer 3 times and processed with
4% paraformaldehyde for 15 minutes, then treated with acidified solution for 5 minutes. Finally, the
micromasses were stained with Alcian blue staining solution (Solarbio, G2541, China) for 30 minutes.

2.6. mRNA Extraction and Quantitative Real-time PCR

According to the manufacturer’s instructions, total RNA was isolated from NPCs by utilizing an EZ-press
RNA Purification Kit (EZBioscience, B004D, USA). The Prime Script RT Master Mix kit (Takara) was used
to perform reverse transcription. On a LightCycler 96 System (Roche), quantitative PCR was carried out by
utilizing the SYBR Green Premix (Accurate Biology, AG11701, China). Ct values were normalized to β-actin as an internal control, and relative expression were computed by using $2^{-\Delta \Delta \text{ct}}$ method. The sequences of the primers are listed as follows: β-actin (forward: 5'-TCTCTGCTCCTCCCTGTTC-3', reverse: 5'-ACACCGACCTTCACCATCT-3'); LRP1 (forward: 5'-ATGTGGCTGTTGAAGGATA-3', reverse: 5'-GCTCGTAGGTGTGATGGTAG-3').

2.7. Proteon Extraction and Western Blot Assay

After washing NPCs with PBS, RIPA lysis buffer (CwBio, CW2333S, China) with 1% protease inhibitor (CwBio, CW2200, China) and 1% phosphatase inhibitor (CwBio, CW2383, China) was added and put them on the ice for 30 minutes. Collected the lysate and centrifuged it at 13000g for 15 minutes. The concentration of protein samples was detected using a BCA kit (CwBio, CW0014, China). Protein samples (30 ug/lane) were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking with 5% skim milk at room temperature for 1 h, the membrane was incubated overnight at 4°C with the following antibodies: LRP1 (1:50000, Ab92544, Abcam), COL2A1 (1:1000, YT1022, Immunoway), Aggrecan (1:1000, ab36861, Abcam), MMP13 (1:1000, GB11247-1, Servicevio), MMP3 (1:1000, YT4465, Immunoway), BCL2 (1:1000, YM3041, Affinity), activated caspase-3 (1:1000, YM3431, Immunoway), NF-κB p65 (1:1000, ab32536, Abcam), Phospho-NF-κB p65 (1:1000, AP0124, Abclonal), and GAPDH (1:5000, A19056, Abclonal). After washing with PBS 3 times, the membrane was incubated with secondary antibody for 1h at room temperature. Enhanced chemiluminescence was used to detect protein bands under completely automated Chemiluminescence imaging system (BLT, GelView 6000 pro, China).

2.8. Flow cytometric analysis

The apoptosis rate was measured by flow cytometry. Both supernatant and cells were collected after treatment with IL-1β and emodin for 48 h. After centrifugation and washing twice with PBS, the NPCs was treated with annexin V/FITC and propidium iodide (PI) according to the instruction of Annexin V Apoptosis Detection Kits (Invitrogen, BMS500FI, USA). The apoptosis rate of NPCs was measured by Flow Cytometer (BD, FACVerse, USA).

2.9. Knockdown of LRP1 by siRNA

One day before transfection, 200000 cells/well were plated in 6-well plates. NPCs were transfected with small interfering RNAs (siRNA; Gene Pharma, China) targeting rat siRNA LRP1-2748 (sense: 5'-GCAAGUGGGUACCACAATT-3', antisense: UUUGUUGGUAC CCACUUGCTT) and siRNA LRP1-3308 (sense: 5'-GCAGAUGCAUAAUACUAATT-3', antisense: 5'-UUGAUGUUAAUGCAUCUGCTT-3') using Lipofectamine 3000 (Invitrogen, L3000015, USA). A negative control (NC) siNC (sense: 5'-UUCUCCGAACGUUGACUCA-3', antisense: 5'-AUCUUGUGCUGUCCGUUGUAG-3') was used as a control. After 48 hours of transfection, the effectiveness of the LRP1 knockdown were evaluated using western blot.
2.10. Immunofluorescence

The NPCs were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking with 10% goat serum (Solarbio, SL038) for 1 h, the cells were incubated with anti-NF-κB p65 (1:200, AF5006, USA) overnight at 4°C. The cells were washed with PBS 3 times and treated with Alexa Fluor 488-labeled goat anti-rabbit/mouse IgG secondary antibody (1:200 dilution) and DAPI for 1 h in the dark environment at room temperature. Images were obtained by inverted fluorescence microscopy (Olympus, IX73, Japan).

2.11. Histology and Immunohistochemistry

After being submerged in xylene 3 times for 10 minutes each time, the paraffin slices were then placed in anhydrous ethanol, 95% ethanol, 85% ethanol, and 75% ethanol for five minutes respectively, then put them in distilled water for five minutes. Hematoxylin and eosin (HE) and safranin O-fast green staining (SO) were carried out according to the instructions of Hematoxylin-Eosin/HE Staining Kit (Solarbio, G1120, China) and Modified Safranine O-Fast Green FCF Cartilage Stain Kit (Solarbio, G1371, China). Antigen retrieval was processed with sodium citrate in a microwave oven at 98°C for 20 minutes. Then, the slides were treated with endogenous peroxidase blocker for 10 min and blocked with normal goat serum for 10 min. The slides were incubated with the primary antibodies at 4°C overnight. Primary antibodies: LRP1 (1:400), COL2A1 (1:100), and BCL2 (1:100). The sections were incubated with biotin-labeled goat anti-rabbit/mouse IgG polymer at room temperature for 15 minutes and treated with HRP-conjugated streptavidin for 10 min. Finally, the sections were treated with DAB solution (ZSGB-BIO, ZLI-9017, China) and then counterstained with hematoxylin. IHC images were obtained under a microscope (Nikon, NI-U, Japan).

2.12. Magnetic resonance imaging (MRI) analysis

The rat tail was scanned by MRI on a Siemens Magnetom Vida 3.0T (Siemens Healthineers, Erlangen, Germany) with the following parameters: TR 2700 ms, TE 70 ms, 0.29× 0.29 mm, thickness 2 mm, FOV 150 × 112.5 mm.

2.13. Statistical Analysis

All data from three independent repeated experiments were analyzed using GraphPad Prism 9 software and are reported as the mean ± standard deviation (SD). Statistical significance was determined using Student’s t-test or one-way ANOVA of variance with Tukey’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001 was considered statistically significant and ns was considered no significant.

3. Results

3.1. Emodin promote anabolism and inhibit catabolism of ECM in NPCs
To better investigate the effect of emodin on NPCs, we tested the toxicity of emodin on NPCs. As shown in Fig. 1a and 1b, after 24 h of stimulation with emodin, there was no obvious toxicity except 80 µM (Fig. 1A). After 48 hours of stimulation, there was no obvious toxicity at concentrations of 2.5, 5 or 10 µM (Fig. 1B). IL-1β was used to induce cell degeneration model in vitro. High-density culture and Alcian blue staining were used to figure out the impact of emodin on matrix secretion in vitro. The results showed that IL-1β group had less Alcian blue staining than the NC group (Fig. 1c), which indicated that ECM secretion from NPCs was decreased. Emodin at concentrations of 5, 10, 20 µM increased Alcian blue staining compared to that in the IL-1β group (Fig. 1C), indicating that emodin effectively improved the ECM secretion under stimulation of IL-1β. Although the concentration of 20 µM emodin was also effective, it was excluded considering its cytotoxicity. Based on the results above, emodin at concentration of 5 and 10 µM were used for the in vitro experiments. The western blot (WB) results revealed 10 µM emodin significantly downregulated IL-1β-induced MMP3 and MMP13 expression and upregulated the expression of COL2A1 and aggrecan (Fig. 1D,F-I). Immunofluorescence (IF) further verified the results of WB (Fig. 1E). Based on the results above, emodin promoted matrix synthesis and inhibit matrix degradation in NPCs.

3.2. Emodin alleviates apoptosis induced by IL-1β in NPCs

We further investigated the effect of emodin on apoptosis. WB analysis illustrated that 10 µM emodin upregulated the expression of antiapoptotic protein BCL2 and downregulated the expression of proapoptotic protein cleaved-capsase3 in IL-1β-treated NPCs (Fig. 2A-C). However, 5 µM emodin had little effect on apoptosis, which was consistent with its effect on ECM metabolism. Moreover, the results of the flow cytometry detection and IF also demonstrated that 10 µM emodin significantly rescued IL-1β-induced apoptosis (Fig. 2D-F), which further verified the results of WB.

3.3. Emodin inhibits NF-κB signaling pathway

Previous studies have demonstrated that the activation of NF-κB exacerbates the progression of IDD. It induces matrix degradation and promotes apoptosis of NPCs. Hence, we explored whether emodin inhibits the NF-κB pathway. WB results showed that the protein level of p-p65 was time-dependently increased in IL-1β-treated NPCs. After treatment with emodin, the activation of p-p65 was time-dependently inhibited (Fig. 3A-B). In addition, the immunofluorescence results showed that emodin substantially inhibited IL-1β-induced nuclear translocation of p65 (Fig. 3C-D), indicating that emodin can inhibit the NF-κB efficiently.

3.4. Emodin upregulates LRP1 protein level to inhibit NF-κB

LRP1 was reported to negatively regulate inflammation and apoptosis in many diseases, such as osteoarthritis and neurodegenerative disease. LRP1 was also reported to inhibit the NF-κB pathway. We wanted to determine whether emodin affects the expression of LRP1 and whether inhibition of NF-κB is regulated by LRP1. qPCR illustrated that IL-1β downregulated the mRNA level of LRP1, but both
concentration of 5 µM and 10 µM had no effect on LRP1 mRNA levels (Fig. 4a). However, WB analysis showed that 10 µM emodin significantly increased the protein level of LRP1 (Fig. 4B-C), which indicated that emodin involves in post-translation regulation of LRP1. Then, we found LRP1 protein level was increased in the emodin-treated group under treatment with CHX, which was consistent with the MG132-treated group (Fig. 4D-G), indicating that emodin increased the LRP1 protein level by inhibiting protein degradation in proteasome pathway. Next, siRNA LRP1 was used to investigate if LRP1 regulates the inhibition of NF-κB. First, we explored the knockdown efficiency of two siRNA LRP1 sequences, WB analysis showed LRP1 was successfully knocked down by siRNA LRP1 (Fig. 4H-I). We selected the siLRP1-2748 for the following experiment. The results of WB and IF showed the effect of emodin on p-p65 and nuclear translocation of p65 were reversed after knockdown of LRP1 (Fig. 4J-M), indicating that emodin inhibit NF-κB pathway by LRP1.

3.5. Knockdown of LRP1 inhibits the effect of emodin on matrix metabolism and apoptosis

To further explore the effect of LRP1 on matrix metabolism and apoptosis regulated by emodin. WB results illustrated that emodin upregulated the protein level of COL2A1, Aggrecan and BCL2 in IL-1β-treated NPCs, and downregulated MMP13 and cleaved-caspase3 protein level at the same time, but these results were reversed after knockdown of LRP1 (Fig. 5A-F). Alcian blue staining further verified the results of WB (Fig. 5G). Therefore, emodin alleviated the metabolic imbalance of ECM and apoptosis, at least, partly through LRP1.

3.6. Emodin ameliorates IDD in the rat model

To further explore the therapeutic effect of emodin in animals, after establishing the model of intervertebral disc degeneration, we injected emodin (10 µM) into the intervertebral disc for 4 weeks to observe whether the IDD was improved. Based on the findings of the MRI, the degree of degeneration in the emodin group was less severe than IDD group, and T2-weighted signal intensity was stronger in the emodin group than in the IDD group (Fig. 6A). HE and SO staining showed that the height of the intervertebral disc was lost, and the volume of nucleus pulposus tissue was decreased and replaced by fibrous tissue. However, these results were significantly ameliorated in the emodin group (Fig. 6B). The histological grade analysis revealed that emodin treatment substantially relieved IDD (Fig. 6C). Moreover, immunohistochemical staining (IHC) demonstrated that the expression levels of LRP1, COL2A1, and BCL2 were considerably higher in the emodin group than the IDD group (Fig. 6D-G). We also verified the effect of LRP1 on matrix metabolism and apoptosis regulated by emodin in vivo. IHC results showed the effects of emodin on COL2A1 and BCL2 in vivo were inhibited by siLRP1 (Fig. 7H-J).

4. Discussion

One of the most important contributors of IDD is inflammation[8, 25, 26]. Cytokines such as IL-1β and tumor necrosis factor-α (TNF-α) participate in the inflammatory response in the process of IDD and
induces the production of more inflammatory factors[27], forming a positive feedback pathway to amplify inflammation, downregulating the expression of matrix synthesis genes and upregulating the expression of matrix metalloproteinases, thus aggravating IDD[28, 29]. Emodin, a component of traditional Chinese medicine rhubarb, has been found to have anti-inflammatory properties. Here, we investigated its impact in an IL-1β environment that induces IDD. Our research showed emodin inhibited matrix degradation and apoptosis in IL-1β-treated NPCs, which was achieved, at least, by inhibiting activation of NF-κB. Furthermore, it can increase LRP1 protein level by inhibiting degradation through proteasome pathway. However, these beneficial effects were reversed by siLRP1, indicating that emodin exerted its protective effect via suppressing NF-κB in an LRP1-dependent manner.

Emodin as a traditional Chinese medicine dates back more than two thousand years. Emodin is widely used in a variety of inflammatory diseases for its anti-inflammatory functions. Studies have shown that emodin reduces pulmonary edema and inflammation in the lung tissue of rats with acute pancreatitis, downregulates TNF-α and IL-1β, and increases claudin-4, claudin-5, and occludin expression in lung tissue[30]. Moreover, emodin substantially decreased allergen-induced inflammation in asthmatic mice by inhibiting the T-helper 2 (Th2) immune response, suppressing MMP-9 expression, and increasing HO-1 expression[31]. In addition, emodin can inhibit the proliferation of synovial cells in rheumatoid arthritis induced by IL-1β and endotoxin[32]. Matrix metalloproteinases (MMPs) have the capability of destroying various extracellular matrix protein such as Aggrecan and type II collagen[21]. MMP3 and MMP13 are reported to be the main factors leading to ECM destruction in the IVD[33]. Considering that MMPs can be upregulated by cytokines such as IL-1β and TNF-α[34], We want to ensure whether emodin can mediate MMPs expression under stimulation of IL-1β. Our study showed that emodin (10 µM) rescued the COL2A1, Aggrecan, MMP3 and MMP13 expression under IL-1β environment, which is consistent with previous findings of emodin in osteoarthritis[35, 36]. However, emodin at concentration of 5 µM did not influence COL2A1, Aggrecan and MMP3 expression, which also explained why Alcian blue staining was lower in the 5 µM group than in the 10 µM group. This study showed that emodin can alleviated matrix metabolism disorder under inflammation, which was not been studied.

Additionally, apoptosis is a key contributor to IDD. In progression of IDD, Apoptosis reduces the number of NPCs and weakens their ability to secrete matrix. When the number of NPCs decreases further, nucleus pulposus tissue loses water and is replaced by fibrous tissue, which makes IVD more difficult to repair. Inflammation is an important factor resulting in apoptosis[37]. For example, inflammatory factors upregulate the level of intracellular ROS, induce mitochondrial dysfunction and activation of NLRP3 inflammatory bodies, downregulating the expression of the anti-apoptosis gene BCL2, and upregulating the cleaved caspase-3[38]. In addition, inflammatory factors can induce endoplasmic reticulum stress which is another important pathway leading to apoptosis[39]. In a model of cerebral ischemia-reperfusion damage, emodin reduces ROS generation and neuronal apoptosis through activating the ERK-1/2 signaling pathway[40]. A recent study has shown that emodin can inhibit IL-1β-induced ROS and inhibit NF-κB to reduce apoptosis of NPCs. However, in our study, we found that emodin inhibits NF-κB by upregulating the protein level of LRP1 to reduce apoptosis of NPCs, and emodin can also reduce apoptosis to alleviate IDD in the rat model, which has not been done in previous study.
Matrix metabolism and apoptosis are related to NF-κB pathway[41], which has been proven to be the pathogenic factor of IDD[42, 43]. NF-κB can promote the expression of various inflammatory genes, such as MMPs, IL-1β, TNF-α, IL-6 and iNOS. In turn, these inflammatory factors continue to activate NF-κB and downstream effectors to amplify the inflammatory response[34]. Therefore, NF-κB has become an important intervention target for IDD. However, whether emodin inhibiting NF-κB in IDD remains unclear. Our findings demonstrated that emodin inhibited the production of p-p65 in a time-dependent manner and prevented translocation of p65 into the nuclear, indicating that emodin inhibited NF-κB activation efficiently, which was consistent with previous studies on other diseases[36]. However, the mechanism that emodin inhibits NF-κB in IDD remains unclear. Many researches have reported that LRP1 can inhibit the activation of NF-κB[22, 44–46]. Furthermore, a study showed that emodin upregulates the expression of LRP1[47]. Therefore, we speculated that emodin may regulate LRP1 and thus inhibit NF-κB in IDD. In our study, we found that emodin upregulated the protein level of LRP1 but had no effect on the mRNA level. Further study showed that both emodin and MG132 upregulated the protein level of LRP1 in CHX-treated NPCs, indicating that emodin can inhibit the degradation of LRP1 via the proteasome pathway, which was not found in a previous study[47]. To further verify our hypothesis, we knocked down LRP1 in NPCs. Interestingly, the effect of emodin on NF-κB was eliminated, and the matrix metabolism phenotype and apoptosis phenotype were reversed, which indicated LRP1 is necessary for emodin on treatment of IDD. Nevertheless, the concentration of 5 µM did not upregulate the protein level of LRP1, which explained why 5µM emodin have little effect on IDD. Moreover, emodin successfully alleviated IDD in rat model. In addition, we also confirmed that the effect of emodin on the expression of COL2A1 and BCL2 was cancelled after siLRP1 in vivo, which was consistent with in vitro results. However, there are still several limitations to our study. We haven't explored how LRP1 inhibits NF-κB. In addition, we did not explore the potential role and mechanism of LRP1.

In conclusion, this study showed that emodin therapeutic effect on IDD by inhibiting matrix metabolism disorder and apoptosis through LRP1 in vitro and in vivo, which was related to LRP1-mediated inhibition of NF-κB (Fig. 7). This study provided evidence for emodin as a potential drug for the treatment of IDD.

**Abbreviations**

LBP
Low back pain
IDD
Intervertebral disc degeneration
IVD
Intervertebral disc
ECM
Extracellular matrix
NPC
Nucleus pulposus cell
NC
Negative control
NF-κB
Nuclear factor kappa-B
MMPs
Matrix metalloproteinases
MMP3
Matrix metalloproteinase 3
MMP13
Matrix metalloproteinase 13
COL2A1
Collagen type 2
p-p65
Phosphorylated P65
IL-1β
Interleukin-1
BCL2
B cell lymphoma 2
LRP1
Low density lipoprotein receptor related protein 1
CHX
Cycloheximide
HE
Hematoxylin and eosin
SO
safranin O–fast green
MRI
Magnetic resonance imaging
PBS
Phosphate-buffered saline
ROS
Reactive oxygen species
TNF-α
Tumor necrosis factor-α
NLRP3
NOD-like receptor thermal protein domain associated protein 3

Declarations

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Declaration of interest

The authors disclose no potential conflicts of interest.

Declaration of competing interest

The authors disclose no potential conflicts of interest.

Author's contribution


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Availability of data and materials

The data supporting the findings of this study are available from the corresponding author on reasonable request.

References


Figures
Figure 1

Emodin promote anabolism and inhibit catabolism of ECM in NPCs

A-B, CCK-8 was used to determine the cell viability of NPCs treated with emodin at dose of 2.5, 5, 10, 20, 40, 80 μM for 24 and 48 hours. NPCs incubated with only medium served as controls and were deemed 100% viability (n=3). C, micromasses of NPCs were cultivated in DMEM containing 2% FBS and 1% ITS.
and treated with IL-1β at 40 ng/mL alone or IL-1β combined with various concentrations of emodin for 5 days. Alcian blue staining was carried out to assess ECM secretory ability of NPCs (n=3). D, F-I, after pretreatment with emodin at 5 and 10 μM for 2 h, IL-1β was added to NPCs for 48 h. The protein levels of COL2A1 (n=3), Aggrecan (n=4), MMP3 and MMP13 were detected by western blot (n=3). E, immunofluorescence analysis of COL2A1 in NPCs after pretreatment with emodin at 10 μM for 2 h and treatment with IL-1β for 48 h, (bar=20 μm). The data are displayed as the mean ± SD. ns: no significance; *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 2

Emodin alleviates apoptosis induced by IL-1β in NPCs

A-C, after pretreatment with emodin at 5 and 10 μM for 2 h, then IL-1β was added to NPCs for 48 h. The protein levels of BCL2 (n=3) and cleaved caspase-3 (n=4) were measured by western blot. D-E, emodin at 10 μM was given to NPCs under IL-1β stimulation for 48 h. The rate of apoptosis was evaluated by flow cytometry (n=5). F, immunofluorescence analysis of cleaved-caspase3 in NPCs after pretreatment with emodin at 10 μM for 2 h and treatment with IL-1β for 48h (n=3), (bar=20 μm). The data are presented as the mean ± SD. ns: no significance; *p < 0.05; **p < 0.001; ***p < 0.0001.
Figure 3

Emodin inhibits NF-κB signaling pathway

A-B, western blot analysis of p-p65 and total p65 expression in NPCs with pretreatment of emodin at 10 μM in the IL-1β + EM group for 1 h followed by treatment with IL-1β for 0, 15, 30, 60, and 120 min in the IL-1β group and IL-1β + EM group (n=3). C-D, immunofluorescence analysis of nuclear p65 in NPCs after
pretreatment with emodin at 10 μM for 2 h and treatment with IL-1β for 2 h (n=3), (bar=20 μm). The data are presented as the mean ± SD. ns: no significance; *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 4

Emodin upregulates LRP1 protein level to inhibit NF-κB
A, gene expression analysis of LRP1 in NPCs after treatment with IL-1β and IL-1β+EM for 24 h (n=3). B-C, western blot analysis of LRP1 expression in NPCs with pretreatment of emodin at 5 μM and 10 μM for 2h followed by treatment with IL-1β for 48 h (n=4). D-G, western blot analysis of LRP1 expression in NPCs pretreated with CHX at 200 μg/mL for 2 h followed by treatment with MG132 at 10 μM or emodin at 10 μM for 6 h (n=4). H-I, western blot analysis of the LRP1 protein level in NPCs with treatment of siNC and siLRP1 for 48 h. J-K, western blot analysis of p-p65 and p65 expression in NPCs treated with siNC in the NC, IL-1β, and IL-1β+EM groups and siLRP1 in the IL-1β + EM group for 24 h followed by pretreatment of emodin at 10 μM and the treatment with IL-1β for 24h (n=3). L-M, immunofluorescence analysis of the nuclear translocation of p65 after treatment with siNC in the NC, IL-1β, and IL-1β + EM groups and siLRP1 in the IL-1β + EM + siLRP1 group for 48h followed by pretreatment with emodin for 2h and then treatment with IL-1β for 2h (n=3), (bar=20). The data are presented as the mean ± SD. ns: no significance; *p < 0.05; **p < 0.01; ***p < 0.001.
Knockdown of LRP1 inhibits the effect of emodin on matrix metabolism and apoptosis

A-F, after siNC and siLRP1 treatment for 24 h, IL-1β and emodin at 10 μM were added to stimulate NPCs for 48 h. Relative expression levels of COL2A1, Aggrecan (n=3), MMP13 (n=4), BCL2 (n=3) and cleaved caspase-3 (n=4) detected by western blot analysis. G, after treatment with siNC and siLRP1 for 24 hours,
the cells were collected and reseeded as micromasses in a 24-well plate, and then IL-1β and emodin were added for 5 days. Matrix secretion ability was measured by Alcian blue staining. The data are presented as the mean ± SD. ns: no significance; *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 6
Emodin ameliorates IDD through LRP1 in the rat model
A, MRI scanning of the rat caudal intervertebral discs after injection with emodin for 1 month (n=6). B, HE and SO staining of intervertebral discs after injection with emodin for 1 month (upper panel, bar=500 μm; lower panel, bar=100 μm). C, Histological grading scores (HE) of the annulus fibrosus and nucleus pulposus (n=3). D, Immunohistochemistry analysis of LRP1, COL2A, and BCL2 expression in the intervertebral discs of rats as shown after 1 month after injection with emodin (upper panel, bar=50 μm; lower panel, bar=10 μm). E-G, Statistical analysis of the average optical density (AOD) of the LRP1-, COL2A1-, and BCL2-positive staining area (n=3). H, Immunohistochemical staining of COL2A1 and BCL2 (upper panel, bar=50 μm; lower panel, bar=10 μm). I-J, statistical analysis of the average optical density (AOD) of the COL2A1-, and BCL2-positive staining area (n=3). The data are presented as the mean ± SD. ns: no significance; *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 7

Schematic illustration of the possible mechanism of emodin in alleviating IVDD

Emodin upregulate LRP1 protein level by inhibiting its degradation to inhibit NF-κB signaling pathway, thus alleviating apoptosis and matrix metabolism disorder. The picture was drawn through BioRender.