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Dioscin Attenuates Interleukin 1 β (IL-1 β)-Induced Catabolism and Apoptosis via Modulating the Toll-Like Receptor 4 (TLR4)/Nuclear Factor kappa B (NF- κ B) Signaling in Human Nucleus Pulposus Cells

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Data Interpretation D
Manuscript Preparation E
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Background: Nucleus pulposus (NP) cell dysfunction and apoptosis contribute to disc degeneration. Dioscin, a natural steroid saponin, has been demonstrated to have anti-inflammatory, antiapoptotic, and antioxidative effects in various diseases. However, little is known about the roles of dioscin in intervertebral disc degeneration.


Material/Methods: To evaluate the roles of dioscin in disc degeneration and its specific mechanism, human NP cells were incubated with IL-1 β and various concentrations of dioscin. Cell viability, extracellular matrix protein expression, catabolic factors, degree of apoptosis, inflammatory factors, and related signaling pathways were evaluated by western blotting, fluorescence immunostaining, TUNEL staining, and reverse transcription PCR.

Results: Dioscin inhibited IL-1 β -activated apoptotic signaling and catabolic activity in NP cells. Dioscin suppressed TLR4/NF- κ B signaling, and attenuated the level of inflammatory mediators (IL-6, TNF- α) in IL-1 β -stimulated human NP cells.

Conclusions: Our work provides the first evidence that dioscin attenuates IL-1 β -activated inflammation and catabolic activity in human NP cells through inhibiting the TLR4/NF- κ B pathway, indicating that dioscin is a new potential candidate for clinical therapy to attenuate disc degeneration.

MeSH Keywords: **Apoptosis • Inflammation • Intervertebral Disc Degeneration • Toll-Like Receptor 4**

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Background

The intervertebral disc is an important component of the spinal structure, which absorbs, alleviates, and transmits loads on the human body and increases the mobility of the spine [1]. However, disc decompensation under certain conditions can lead to progressive destruction of its structure, resulting in disc herniation, discogenic pain, and other disc degeneration-related diseases (e.g., intervertebral disc disease [IDD]), which impose a serious burden on patients' lives as well as socio-economic development. It has been estimated that more than half of the population will suffer from low back pain at different stages of life [2], and more than 40% of cases of low back pain are caused by disc degeneration [3]. Research on disc degeneration has mainly focused on the changes in metabolites during the stress process of the intervertebral disc itself. It is believed that the structure of the intervertebral disc can be destroyed under stimulation by stress factors, such as abnormal stress and nutritional dysfunction, which can lead to cell metabolic disorders, inflammation, apoptosis, autophagy, and other phenomena, eventually leading to disc degeneration [4,5]. However, the pathophysiological mechanism of IDD is unclear, necessitating further research to develop effective preventive and treatment strategies.

Various pathological events, such as mechanical stress, structural deformities, genetic factors, infection, and smoking, activate pro-inflammatory signaling pathways in disc cells. These initializing triggers cause morphological changes in the disc tissues, thereby triggering further inflammation and degenerative processes. Nucleus pulposus (NP) cells are present in a gelatinous extracellular matrix (ECM) containing collagen II and proteoglycan, which are essential for resistance to compressive axial force of the spine [6,7]. In healthy NP tissue, the NP cell maintains the metabolic balance of ECM, including aggrecan and collagen, with long half-lives [8]. Studies have demonstrated that matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin type I motifs (ADAMTSs) disrupt the balance of ECM metabolism during the pathological process of disc degeneration.

Various pro-inflammatory mediators containing tumor necrosis factor (TNF)- α and interleukin (IL)-1 β have been shown to be significantly up-regulated in degenerative disc tissue in humans [9,10]. TNF- α and IL-6, essential pro-inflammatory factors, have been shown to be closely related to the progression of IDD [11]. TNF- α can trigger inflammation, leading to neural swelling and neuropathic pain, and aggravate cell apoptosis due to its cytotoxic effects in humans with lower lumbar pain. IL-1 β , the most studied proinflammatory factor, has been shown to trigger the level of various proinflammatory mediators, including TNF- α , IL-6, and several matrix-degrading enzymes, disrupting the balance of ECM metabolism and

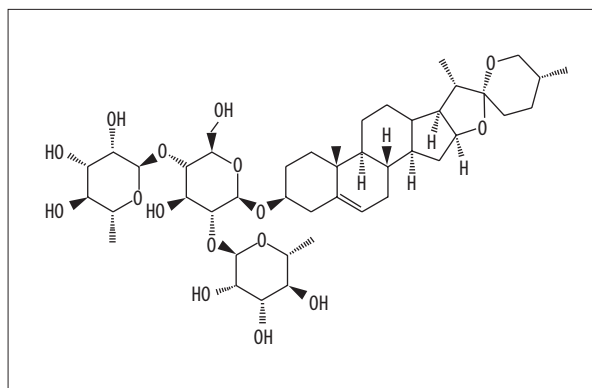


Figure 1. The molecular structure of dioscin.

impairing its turnover in the intervertebral discs [12]. Several molecular pathways, such as nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling, have been shown to be major moderators of IL-1 β -induced inflammation and catabolism [13].

Dioscin, a natural steroid saponin, is extracted from several herbal medicines [14]. Pharmacological studies have suggested that dioscin has anti-inflammatory, anti-apoptotic, and anti-oxidative effects in various diseases [15–19]. For instance, dioscin was shown to attenuate dimethylnitrosamine-induced acute liver injury by inhibiting apoptosis via reductions in the expression of proapoptotic proteins [16]. Dioscin inhibited hepatocellular carcinoma via regulation of apoptosis, autophagy, and DNA damage [20] and it attenuated intestinal ischemia/reperfusion injury through regulating miR-351-5p-related oxidative stress, inflammation, and apoptosis [21,22]. Dioscin markedly prevented non-alcoholic fatty liver disease via regulating lipid metabolism by activating SIRT1/AMPK signaling [23]. Dioscin inhibited systemic inflammatory response syndrome via adjusting the TLR2/NF κ b signaling pathway [24]. Dioscin was also reported to attenuate cerebral ischemia/reperfusion injury via inhibiting TLR4 signaling [25].

However, little is known about the potential effects of dioscin on IDD. Therefore, this study aimed to explore the potential effects of dioscin in IL-1 β -treated NP cells and its specific mechanism of action.

Material and methods

Chemicals and materials

Dioscin (Figure 1) with a purity >99% was purchased from Shanghai Tauto Biochemical Technology Co., Ltd. (Shanghai, China). IL-1 β was acquired from R&D Systems (St. Paul, MN, USA). Antibodies were acquired from Santa Cruz (TLR4, P65, and I κ B α ; Santa Cruz, CA, USA) and Cell Signaling Technology

Table 1. Primers of targeted genes.

	Forward	Reverse
MMP3	CTGGCCTGCTGGCTCATGCTT	GCAGGGTCCTTGGAGTGGTCA
MMP13	AGCCACTTTATGCTTCTCTGATG	GATGTTTAGGGTTGGGGTCTTC
ADAMTS4	CCTGGCAAGGACTATGATGCTGA	GGGCGAGTGTGGTCTGG
ADAMTS5	CGCTCTCTGGCTGTCTTTGAC	AGCAGTGGCTTTAGGGTGTAGC
Collagen II	CTCAAGTCGCTGAACAACCA	GTCTCCGCTCTTCCACTCTG
Aggrecan	GTCAGATACCCATCCCACTC	CATAAAAGACCTCACCTCCAT
IL-6	ATGAACTCCTTCTCCACAAGC	CTACATTTGCCAAGAGCCCTCAGGCTGGACTG
TNF- α	AGGCGGTGCTTGTCTCTCA	GTTGCGAGAAGATGATCTGACTGCC
β -actin	ATTGCCGACAGGATGCAGAA	GCTGATCCACATCTGCTGGA

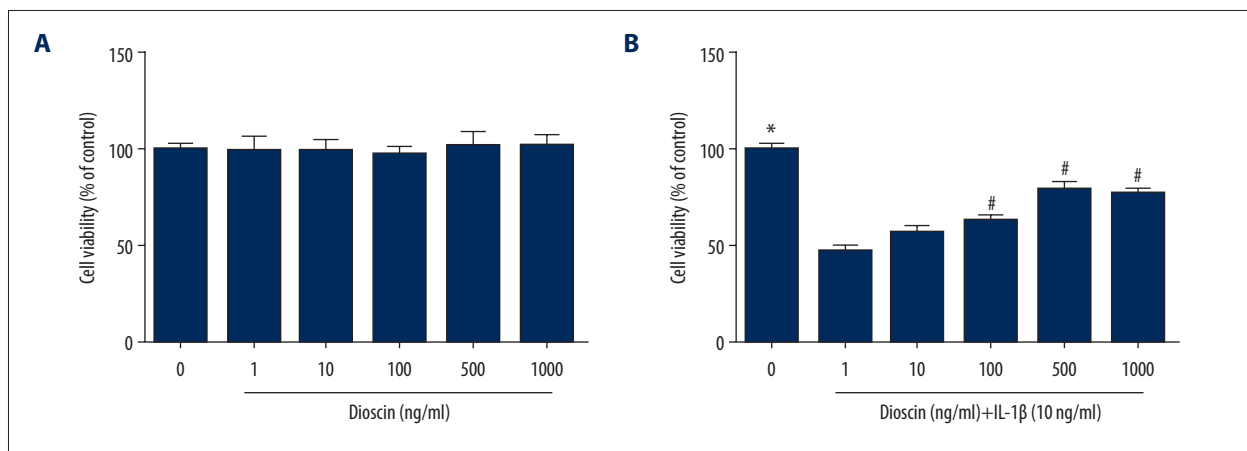


Figure 2. Effects of dioscin on IL-1 β -activated cell viability in human NP cells. (A) The cytotoxic effects of dioscin on human NP cells were examined at different concentrations by the CCK-8 method. (B) CCK-8 analysis of dioscin-treated human NP cells stimulated by IL-1 β . The results are presented as the means \pm SD. * $P < 0.05$ relative to the control group; # $P < 0.05$ relative to the IL-1 β group, $n = 5$.

(collagen II, cleaved caspase-3, caspase-9, Bcl-2, and β -actin; Beverly, MA, USA). Other reagents not mentioned here were acquired from Sigma-Aldrich.

Primary human NP cell culture and treatment

Human NP tissue collection conformed to the guidelines of the Ethics Committee of the Second Affiliated Hospital of Hainan Medical University. Informed consent for disc tissue collection was acquired from patients or their relatives. The protocol of human NP cell culture was performed as described previously [26]. NP cells at passage 1–2 were prepared for reverse transcription PCR, immunofluorescence assay, and western blotting. The cells were planted into 6-well plates. After 2 days, the samples were treated with dioscin (1, 10, 100, 500 ng/mL) supplemented with IL-1 β (10 ng/mL) for 1 day, and NP cells were then used for the experiments.

Viability assay

The NP cells were seeded and cultured in 96-well plates (5×10^3 cells/well) for 1 day and incubated with the various concentrations of dioscin with or without IL-1 β (10 ng/mL). After incubation for 1 day, CCK-8 solution (10 μ L) was added to each well for 2 h. The absorbance of samples was tested at 450 nm by a spectrophotometer.

Immunofluorescence assay

NP cells were seeded and cultured on coverslips, treated with 4% paraformaldehyde for 15 min, and then treated in 0.5% Triton X-100 for 10 min. Following incubation in blocking buffer containing 5% BSA for 30 min, samples were treated with the primary antibody at 4 $^{\circ}$ C. The following day, samples were treated with the secondary antibody for 1 h at 37 $^{\circ}$ C. Sample were sealed with VECTASHIELD (Vector Laboratories, Burlingame,

CA, USA) including DAPI. The immunostained samples were imaged by laser scanning confocal microscopy.

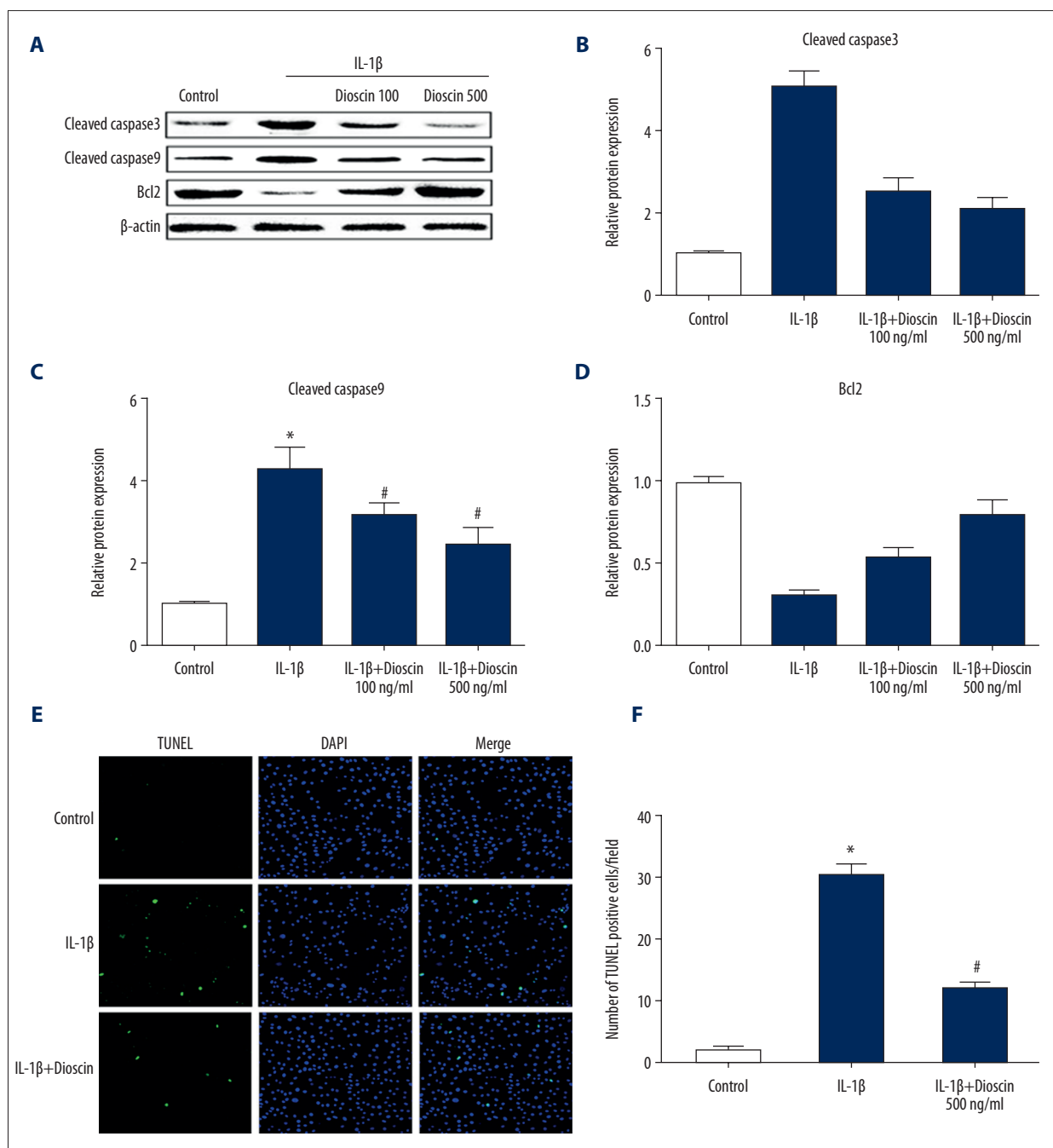
Western blotting

All protein samples were isolated using cell lysis buffer. The concentration of total protein was tested with a BCA protein assay kit. The protein specimens were added onto SDS gel electrophoresis and transferred onto PVDF membrane. After blocking nonspecific binding sites, the membranes were immunoblotted

with primary antibodies. The following day, samples were treated with the secondary antibody for 60 min at 37°C. Band intensity was detected using the ChemiDoc XRS+ Imaging System.

TUNEL assay

After treatment, NP cells were incubated with 4% paraformaldehyde, and then treated in 0.5% Triton X-100 for 10 min. Apoptotic NP cells were stained with an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the



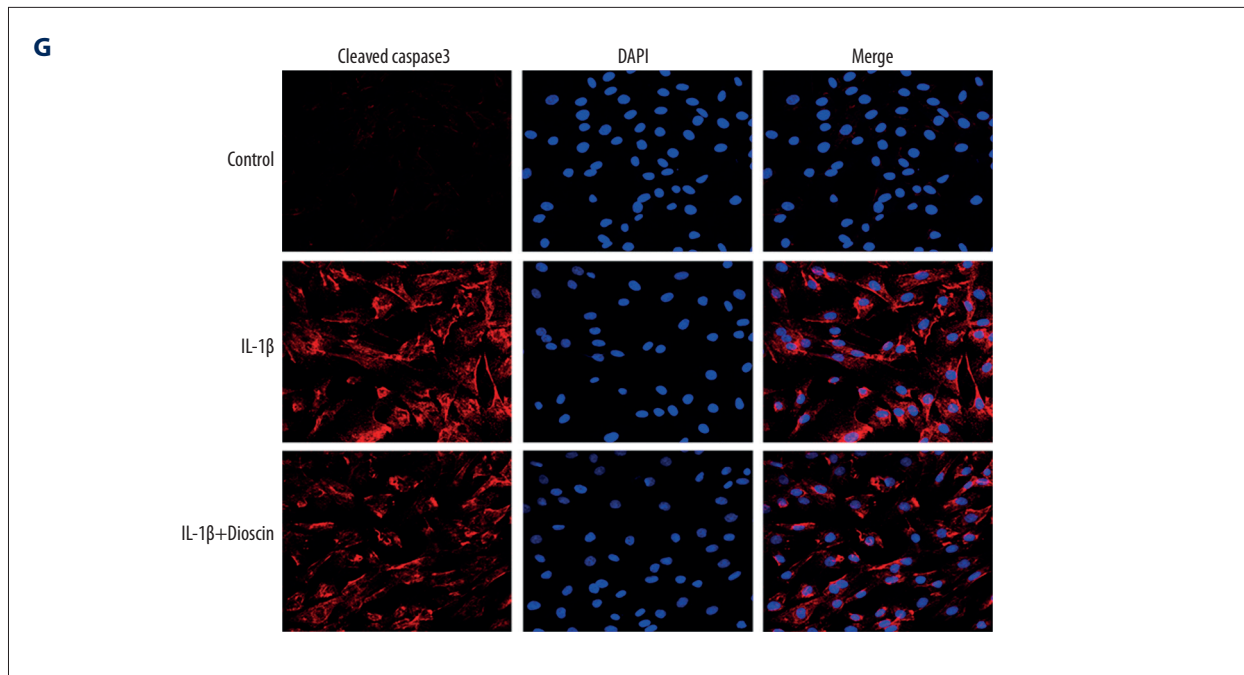


Figure 3. Effect of dioscin on IL-1 β -activated apoptotic activity in human NP cells. (A, B) Apoptotic activity was evaluated using the TUNEL method. (C–F) Western blotting and quantitative analysis of cleaved caspase-3, -9, and Bcl-2 expressions. (G) Fluorescence immunostaining of cleaved caspase-3 (red) and nucleus (blue). The results are presented as the means \pm SD. * $P < 0.05$ relative to the control group; # $P < 0.05$ relative to the IL-1 β group, $n = 5$.

manufacturer's instructions, and DAPI. The degree of apoptotic level in each group was determined by laser scanning confocal microscopy.

Reverse transcription PCR assay

Total RNA of NP cells was extracted using the TRIzol method. cDNAs were synthesized by reverse transcription using oligo (dT) with RNA samples. cDNAs were amplified with a SYBR Green PCR kit. The primers of the targeted genes are listed in Table 1. mRNA expression was quantified using the ddCt method.

Statistical analysis

Results were analyzed as means \pm standard deviations (SD). Differences among groups were tested by one-way analysis of variance and Tukey's post hoc test. $P < 0.05$ was regarded as statistical significance.

Results

Effects of dioscin on IL-1 β -activated cell viability in human NP cells

We first examined the safe dose range of dioscin that did not adversely affect the NP cells viability using the CCK-8 method.

Dioscin exhibited no effects on cell viability at doses of 1 to 1000 ng/mL (Figure 2A). NP cells were incubated with IL-1 β and different concentrations of dioscin (1, 10, 100, and 500 ng/mL). Dioscin showed a dose-dependent inhibitory effect on IL-1 β -induced cell viability (Figure 2B).

Effects of dioscin on IL-1 β -activated apoptotic signaling in human NP cells

To clarify the molecular events related to the roles of dioscin in IL-1 β -activated apoptotic activity, the level of cleaved caspase-3, caspase-9, and Bcl-2 were determined. Treatment with IL-1 β markedly up-regulated the level of cleaved caspase-3 and caspase-9, and down-regulated Bcl-2 expression. However, dioscin dose-dependently attenuated these IL-1 β -induced alterations in level of cleaved caspase-3, caspase-9, and Bcl-2 (Figure 3A–3D). Furthermore, the results of TUNEL staining showed similar anti-apoptotic roles of dioscin in IL-1 β -treated NP cells as was observed in untreated controls (Figure 3E, 3F). The immunofluorescence data of cleaved caspase-3 were consistent with the above results (Figure 3G). These results suggested that dioscin alleviated IL-1 β -activated apoptotic activity in human NP cells.

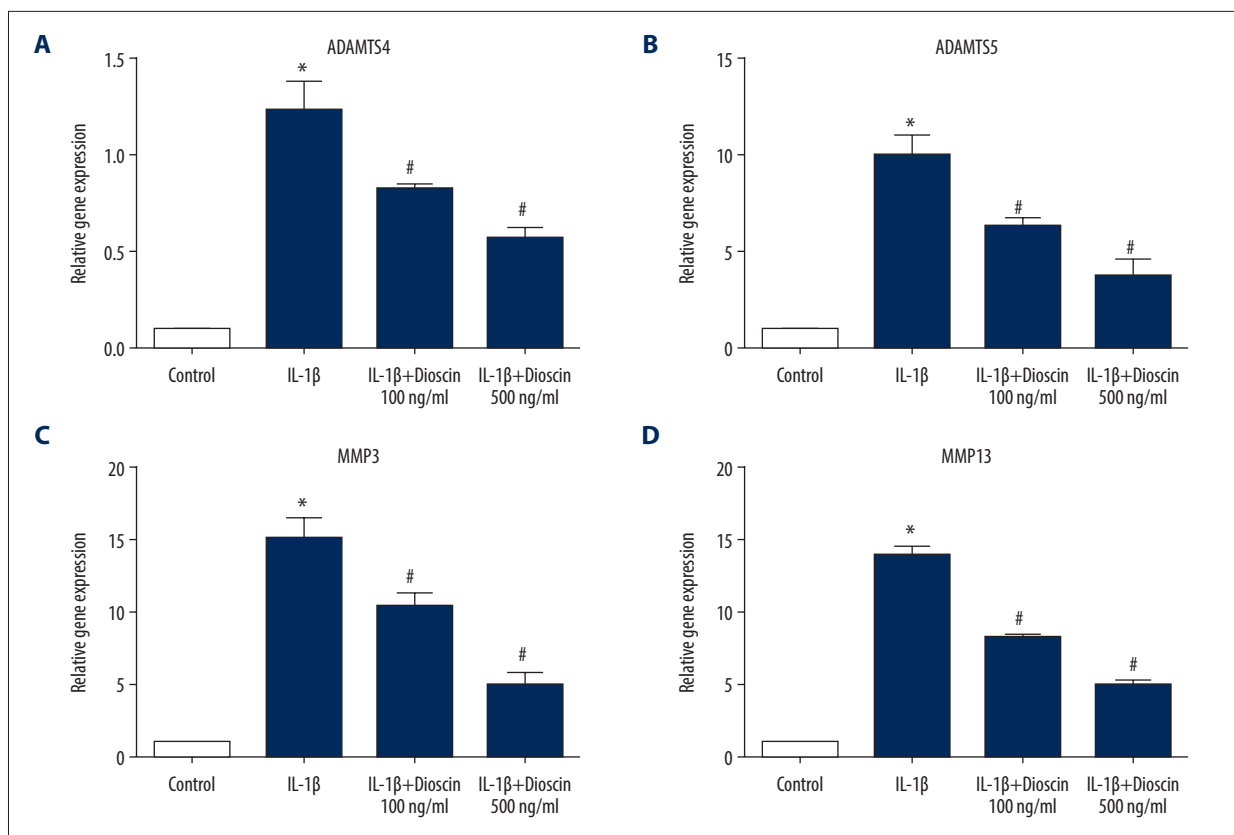


Figure 4. Dioscin suppressed IL-1 β -activated catabolic activity in human NP cells. (A–D) Relative mRNA level of (A, B) ADAMTS4 and ADAMTS5, and (C, D) MMP3, MMP13 were measured by PCR. The results are presented as the means \pm SD. * $P < 0.05$ relative to the control group; # $P < 0.05$ relative to the IL-1 β group, $n = 5$.

Dioscin suppressed IL-1 β -activated catabolic activity in human NP cells

Catabolic enzymes including MMP3 and MMP13 together with ADAMTS4 and ADAMTS5 are closely related to the ECM catabolism of NP cells. Thus, we measured these catabolic factors by PCR to assess the catabolic activity of the NP cells. As shown in Figure 4, the expressions of ADAMTS4 and ADAMTS5 were sharply up-regulated in the IL-1 β group and markedly reversed by dioscin. Similarly, the mRNA expressions of MMP3 and MMP13, which were increased in the IL-1 β group, were markedly attenuated by dioscin. These results showed that dioscin treatment inhibited IL-1 β -activated catabolic activity in human NP cells.

Dioscin attenuated ECM degradation in IL-1 β -stimulated human NP cells

The levels of proteins and mRNAs involved in ECM degradation were measured by PCR and immunofluorescence assay. The levels of collagen II and aggrecan, major components of the ECM, were markedly decreased by IL-1 β (Figure 5A, 5B). However, dioscin-treated NP cells showed higher levels of

collagen II and aggrecan mRNA compared to the IL-1 β -treated cells. The results of immunofluorescence staining for collagen II were consistent with the results of the PCR assays (Figure 5C). These data suggested that dioscin treatment preserved ECM protein in IL-1 β -induced NP cells.

Dioscin inhibited IL-1 β -induced release of pro-inflammatory mediators by regulation of NF- κ B signaling in human NP cells

There is accumulating evidence that inflammation is closely related to disc degeneration. Therefore, we evaluated the mRNA level of the inflammatory factors IL-6 and TNF- α . The PCR results were similar to previous results in that dioscin treatment markedly down-regulated the expressions of IL-6 and TNF- α in IL-1 β -treated cells (Figure 6A, 6B). NF- κ B activity is critical for inflammation. Therefore, we measured the effects of dioscin on NF- κ B activation in the human NP cells. NF- κ B protein expression in the dioscin treated groups was markedly reduced compared to that in the IL-1 β -treated group (Figure 6C, 6E). In addition, IL-1 β -induced degradation of inhibitor of kappa B ($\text{I}\kappa\text{B}\alpha$) was evaluated in the human NP cells; treatment with dioscin significantly inhibited $\text{I}\kappa\text{B}\alpha$ degradation

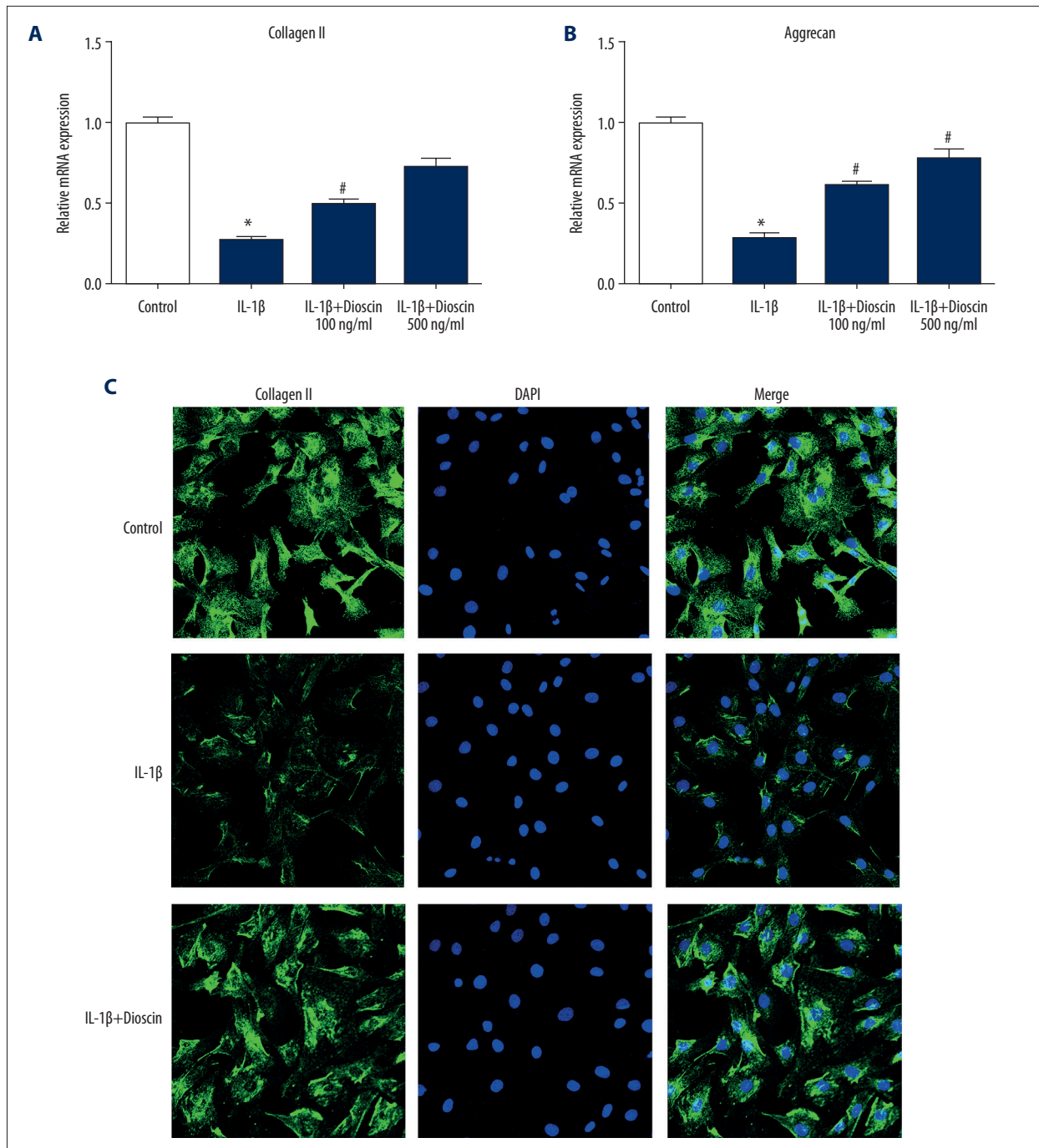


Figure 5. Dioscin attenuated ECM degradation in IL-1 β -stimulated human NP cells. **(A, B)** Relative mRNA level of **(A)** collagen II and **(B)** aggrecan were examined by PCR. **(C)** Fluorescence immunostaining of collagen II protein (green) and nucleus (blue). The results are presented as the means \pm SD. * $P < 0.05$ relative to the control group; # $P < 0.05$ relative to the IL-1 β group, $n = 5$.

in IL-1 β -stimulated human NP cells (Figure 6C, 6F). To explore the effects of the TLR4 pathway on the regulation of NF- κ B signaling, the expression of TLR4 was evaluated. The results suggested that TLR4 level was markedly up-regulated in the IL-1 β treatment group compared to control NP cells, and dioscin treatment reversed these effects (Figure 6C, 6D). Taken

together, these observations indicated that dioscin treatment inhibited inflammation via regulation of TLR4/NF- κ B signaling.

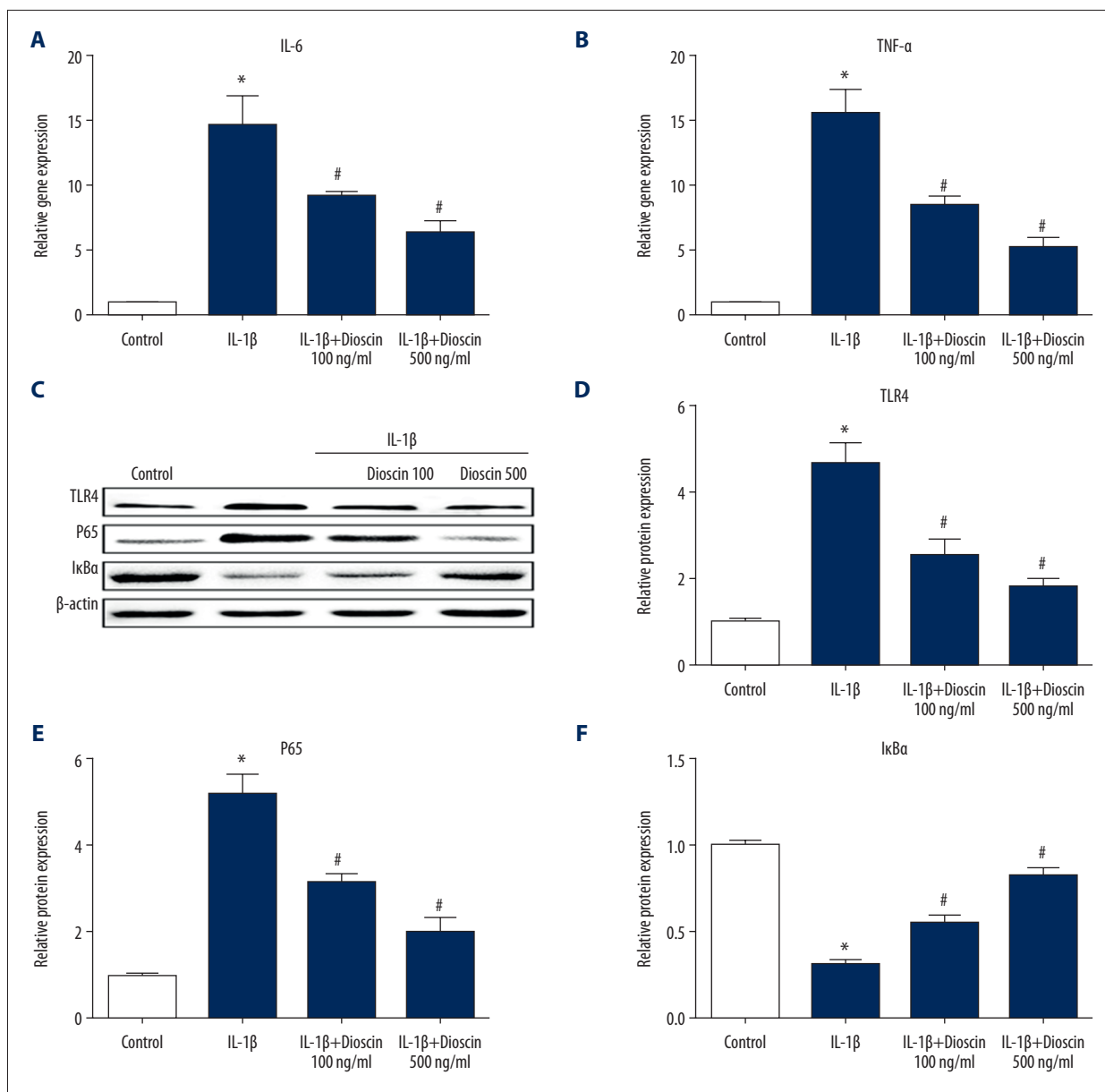


Figure 6. Dioscin suppressed IL-1β-induced release of proinflammatory factors via regulation of the NF-κB pathway in human NP cells. (A, B) Relative mRNA level of (A) IL-6 and (B) TNF-α were examined by PCR. (C-F) Western blotting and quantitative analysis of TLR4, p65, and IκBα. The results are presented as the means ±SD. * *P*<0.05 relative to the control group; # *P*<0.05 relative to the IL-1β group, n=5.

Dioscin inhibited NF-κB translocation to the nucleus in human NP cells

Immunofluorescence assay showed that overexpressed p65 was translocated into the nucleus in NP cells exposed to IL-1β (Figure 7). However, treatment with dioscin significantly suppressed nuclear translocation of p65 in the IL-1β-treated group (Figure 7).

Discussion

Low back pain causes physical disability, and more than 80% of adults worldwide have suffered from low back pain [27]. IDD has been reported to be one of the major reasons for low back pain [28]. Senescence, metabolic imbalance, mechanical stress, and inflammation are involved in the pathogenesis of IDD [29,30]. However, the specific pathological mechanism underlying IDD has not been fully elucidated. The disc tissue consists of the central NP tissue, peripheral annulus

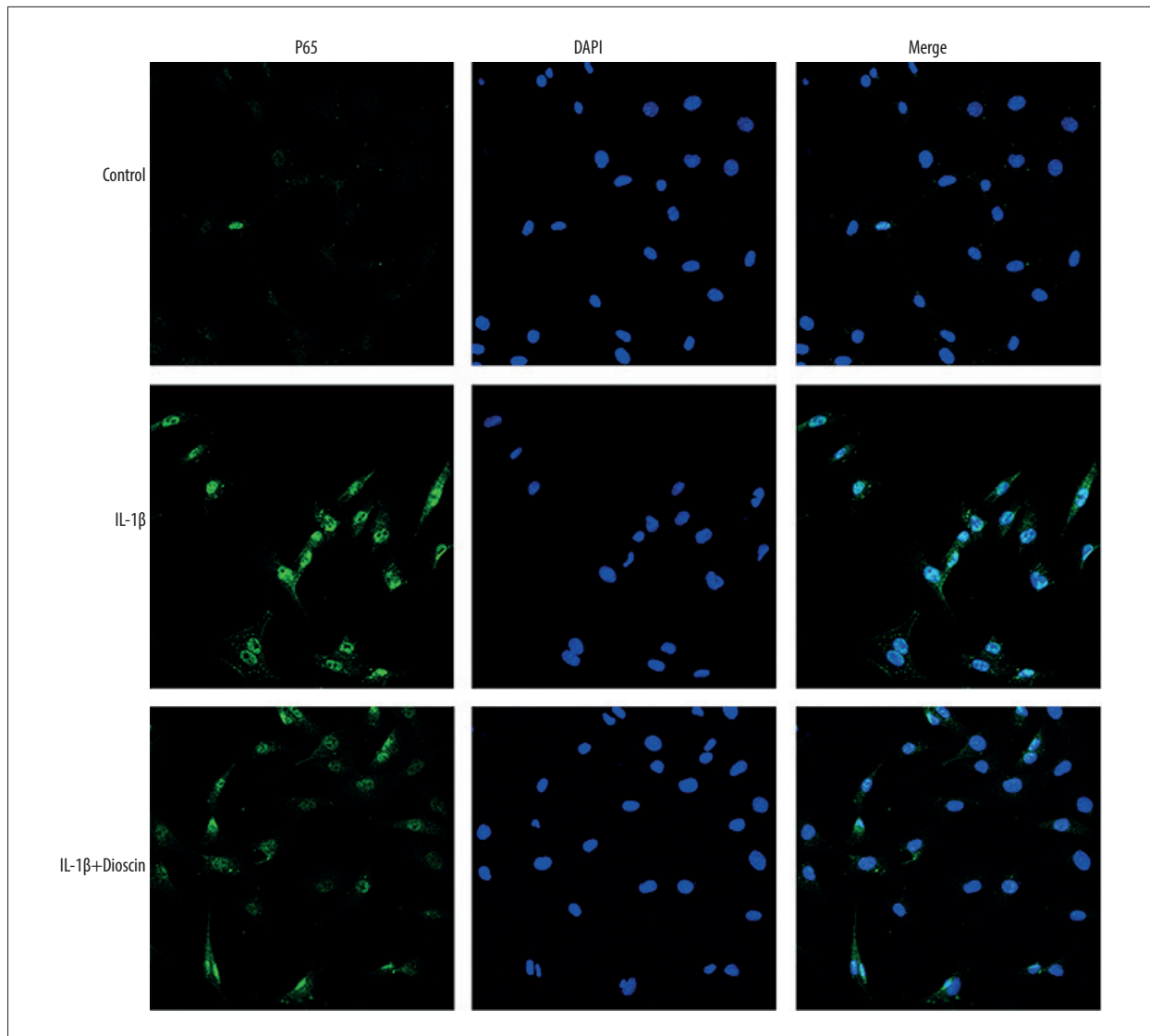


Figure 7. Dioscin inhibited nuclear translocation of NF- κ B in human NP cells. Fluorescence immunostaining of p65 (green) and nucleus (blue).

fibrous tissue, and cartilage endplate. It has been demonstrated that NP cells exhibit a degenerative phenotype in the early stage of IDD [31].

Studies have suggested that inflammation and apoptosis are 2 essential characteristics in NP tissue during the pathological process of IDD [32,33]. In healthy NP tissue, the NP cells maintain the metabolic balance of ECM, including aggrecan and collagen, with long half-lives [8]. A recent study showed that apoptosis of NP cells is closely related to ECM degradation [34]. Furthermore, inflammatory cytokines (IL-1 β and TNF- α) attract death-related signaling complexes through interaction with their specific ligands, and then initiate apoptotic signaling, contributing to DNA fragmentation [35]. It was reported that dioscin protected H9c2 cells against hypoxia/reoxygenation injury by

regulating mitochondrial apoptotic signaling via inhibition of oxidative stress [36]. Dioscin was also shown to attenuate dimethylnitrosamine-stimulated acute liver injury via suppression of apoptotic activity by decreasing the level of FasL, Fas, p53, Bak, caspase-3, and caspase-9 [16]. Our results suggested that IL-1 β promotes NP cell apoptosis, which is partly inhibited by dioscin, suggesting that dioscin exerts an anti-apoptotic effect on NP cells in an inflammatory environment.

It has been reported that the excessive inflammatory response in disc NP tissue is closely related to an increase in the degree of apoptosis of NP cells [37,38]. Therefore, inhibition of the inflammatory response may contribute to the prevention of NP cell apoptosis and reverse degenerative disc changes. TLR signaling pathways were demonstrated to enhance the

expression of inflammatory mediators, such as TNF- α , IL-1, and IL-6, contributing to inflammatory responses [39]. TLR4 was shown to be over-expressed in cartilage during the process of osteoarthritis and to play essential roles in cartilage degeneration [40]. Similarly, *in vitro* and *in vivo* studies indicated that NP cells increased the expression of TLR4 and reacted to TLR4 activation induced by lipopolysaccharide (LPS) administration by enhancing the release of inflammatory factors and reducing the amount of ECM in the intervertebral disc [41]. Ligand binding to TLRs trigger a signaling cascade that contributes to the NF- κ B signaling activation, which increases the expression of inflammatory cytokines and degrading enzymes [42]. LPS can enhance the degradation of ECM protein by binding with TLR4/NF- κ B during the process of rheumatoid arthritis [43]. As a specific ligand of TLR, LPS can enhance the degradation of proteoglycan and aggrecan, and the expression of collagen II by activation of TLR4 signaling in both murine and human articular chondrocytes [44]. IL-1 β can promote inflammatory responses and enhance the release of matrix-degrading enzymes in NP cells [45]. A previous study demonstrated that the level of IL-1 β is markedly up-regulated in NP cells, indicating that IL-1 β may play a critical role in the process of disc degeneration [46]. IL-1 β exerts its inflammatory effects in part by triggering transcription of the inducible nitric oxide synthase gene, leading to the release of high concentrations of nitric oxide [47]. In the present work, treatment with IL-1 β markedly increased the level of TLR4 and activated the

NF- κ B signaling in human NP cells. IL-1 β increased ECM protein degradation via up-regulation of matrix-degrading enzymes. However, dioscin attenuated the TLR4/NF- κ B pathway activation and reversed ECM protein degradation in IL-1 β -stimulated human NP cells. Pharmacological studies have suggested that dioscin exerts an anti-inflammatory effect on various diseases. For instance, dioscin markedly attenuated the release of IL-1 β , IL-6, TNF- α , and p65 via regulation of TLR4/MyD88 signaling to attenuate the inflammatory response [16]. Dioscin was also shown to suppress TNF- α -activated increase of vascular cell adhesion molecule-1 and endothelial lipase via inhibiting the NF- κ B pathway [48].

Conclusions

TLR4/NF- κ B signaling was suggested to be related to the mechanisms of action of dioscin against IL- β -activated inflammatory response, apoptosis, and catabolic activity in human NP cells. Our results provide the first evidence for the pharmacological effects and mechanisms of action of dioscin, which should be considered as a potential new candidate for clinical therapy to attenuate disc degeneration.

Conflict of interest

None.

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