

Original Paper

Hemeoxygenase-1 Suppresses IL-1 β -Induced Apoptosis Through the NF- κ B Pathway in Human Degenerative Nucleus Pulposus Cells

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Key Words

Hemeoxygenase-1 • IL-1 β • Apoptosis • NF- κ B • Human nucleus pulposus cells

Abstract

Background/Aims: Nucleus pulposus cell (NPC) apoptosis is the main factor in intervertebral disc degeneration (IDD); thus, inhibiting the excessive apoptosis of nucleus pulposus cells may be a potential way to alleviate IDD. The effect of Hemeoxygenase-1 (HO-1) on human NPC apoptosis has never been reported. Our study aimed to investigate the effect and mechanism of HO-1 on apoptosis in human degenerative NPCs. **Methods:** Nucleus pulposus tissues were collected from patients with lumbar vertebral fracture (LVF) and IDD. The expression of HO-1 and P65 in intervertebral discs was determined using immunohistochemistry and western blot analysis. Apoptosis of human nucleus pulposus cells was quantified by flow cytometric analysis. A recombinant lentiviral vector overexpressing HO-1 and HO-1-siRNA was used to promote or silence the expression of HO-1 in nucleus pulposus cells. The NF- κ B inhibitor PDTC was used to inhibit the NF- κ B pathway. **Results:** Our study demonstrated that compared with normal samples, IDD samples showed down-regulation of HO-1 expression and up-regulation of P65 expression. Overexpression of HO-1 inhibited the increase in nucleus pulposus cell apoptosis after IL-1 β treatment and simultaneously inhibited the expression of p-P65. Furthermore, after treatment with PDTC, the number of apoptotic cells was significantly decreased with or without overexpression of HO-1. **Conclusion:** HO-1 might play a significant role in IDD, and HO-1 protected degenerative human NPCs against apoptosis induced by IL-1 β through the NF- κ B pathway. These findings would aid in the development of novel therapeutic approaches for IDD treatment.

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Introduction

Many people worldwide suffer from low back pain (LBP), which causes serious socioeconomic problems [1-2]. Many studies have confirmed that intervertebral disc degeneration (IDD) is the leading cause of low back pain. Although the molecular mechanism of IDD has not been fully elucidated, excessive apoptosis of nucleus pulposus cells (NPCs) has been shown to be an important factor in the process of IDD [3-5]. In view of this, inhibition of excessive apoptosis of NPCs may be a novel treatment to postpone the progression of IDD diseases.

Hemeoxygenase-1 (HO-1), the initial rate-limiting enzyme for haeme metabolism, is normally expressed at low levels in various cells. The expression of HO-1 can be increased by stimulation from inflammatory cytokines, heat shock and endotoxins. It can break down pro-oxidant haeme to equimolar amounts of carbon monoxide (CO), biliverdin/bilirubin (BV/BR), and free iron [6]. The role of HO-1 includes antioxidant, anti-inflammatory and anti-apoptotic activities. In addition, it also has protective effects against the development of hypertension, coronary heart disease, diabetes, nephritis and other diseases [7].

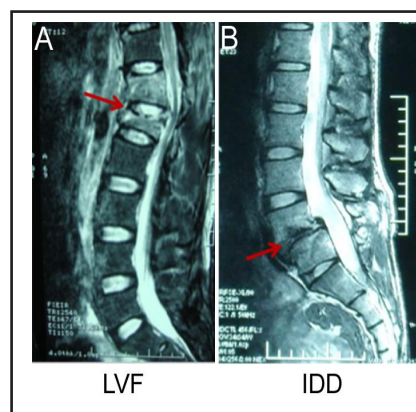
It has been reported that HO-1 can reverse the effect of IL-1 β on human chondrocyte extracellular matrix (ECM) metabolism [8]. Recently, Hu et al. [9] reported that HO-1 can attenuate IL-1 β -induced alteration of anabolic and catabolic activities in human IDD. These results reveal that HO-1 may play a significant role in the progression of IDD. It has been demonstrated that HO-1 prevents non-alcoholic steatohepatitis by suppressing hepatocyte apoptosis in mice [10], and HO-1 can also protect human cardiac stem cells against apoptosis [11]. Furthermore, nuclear factor kappa B (NF- κ B) is a transcription factor that has an important effect on the response of the cell to injury, stress, and inflammatory reactions. Some evidence has indicated that the NF- κ B signalling pathway may play a significant role in NPC apoptosis [12, 13]. However, it is still poorly understood whether HO-1 is associated with NPC apoptosis in human IDD and whether this effect is mediated through the NF- κ B pathway. Hence, we designed an experiment to detect the relationship between HO-1 and apoptosis in human degenerative NPCs and to understand whether the NF- κ B pathway is involved in the protective effect of HO-1 against apoptosis induced by IL-1 β in degenerative human NPCs.

Materials and Methods

Primary NPC isolation and culture

All degenerative NP tissues were acquired from lumbar spine surgery patients. Written informed consent was obtained from all tissue donors before the surgery, and the study protocol was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China). The relatively normal human intervertebral disc (IVD) tissues used as controls were obtained from 6 patients with lumbar vertebral fracture (LVF) who did not have a documented medical history of LBP (2 women and 4 men) (Fig. 1A). The human IDD tissues were obtained from 12 patients with lumbar disc herniation (5 women and 7 men) (Fig. 1B). The degree of IDD was assessed according to the Pfirrmann classification system by using preoperative MRI [14]. Samples from IDD patients were grades III-V, and those samples from LVF patients were grade I.

Fig. 1. Representative lumbar MRIs of one patient with LVF aged 36 years old (A) and another with IDD aged 67 years old (B) were classified according to the Pfirrmann grading system. The red arrows indicate the position of the experimental material.



NP tissues from 12 donors (6 donors per group) were first used for immunohistochemical (IHC) analysis, Western blotting and real-time PCR. NPCs from the IDD patients were isolated by enzymatic digestion and were cultured into a monolayer by incubation in DMEM/F-12 medium (HyClone, USA) supplemented with 20% (V/V) FCS (Gibco, USA), 100 µU/mL streptomycin, and 100 µg/mL penicillin, as described previously [15]. NPCs were incubated at 37°C in an atmosphere containing 5% CO₂. Cells at passage II were used *in vitro* experiments.

IHC Staining

NP specimens were fixed with 4% paraformaldehyde for 24 h and then embedded in paraffin and sectioned at 4 mm for IHC staining. The IHC staining procedure was performed with a streptavidin-peroxidase immunohistochemical kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China) according to the manufacturer's protocol. Briefly, the sections were treated with 3% H₂O₂ for 15 min at room temperature to eliminate endogenous peroxidase activity and were subsequently incubated with 0.125% trypsin for 30 min at 37°C to retrieve the antigen, before being blocked with normal goat serum for 15 min at room temperature. Next, the sections were incubated with rabbit anti-HO-1 (#5853) and rabbit anti-P65 (#8242) primary antibodies overnight at 4°C. Then, the sections were incubated with secondary antibody goat anti-rabbit IgG-HRP (1:5000) and counterstained with haematoxylin.

TUNEL Staining

NP tissues from 12 donors (6 donors from each group) were used for TUNEL staining. DNA strand breaks in dead cells were enzymatically labelled using a TUNEL assay kit (Roche, Switzerland). The sections were treated with 3% H₂O₂ for 15 min at room temperature to eliminate endogenous peroxidase activity, and then the TUNEL reaction solution was added for 1 h at 37°C. Apoptotic NPCs were quantified by counting TUNEL-labelled apoptotic cells, and the results are expressed as counts of TUNEL-labelled apoptotic cells. Stained cells in 3 different areas were counted under a microscope.

Flow cytometry

Apoptosis was detected using an Annexin V/PI apoptosis detection kit (Keygen, China). Briefly, degenerative human NPCs were seeded into six-well culture plates and then treated. In all, 1×10^4 cells were harvested, washed twice with PBS, and resuspended in binding buffer. Cells were incubated with 1.25 µL of annexin V-FITC in the dark at room temperature for 15 min, and flow cytometry was performed immediately. The apoptotic rate is expressed as the sum of the percentage of early (Annexin V+/PI-) and late apoptotic cells (Annexin V+/PI+).

Transfection

A recombinant lentiviral vector overexpressing HO-1 (LV-HO-1) was generated by GeneChem (Shanghai, China). Negative control siRNA (NC-siRNA) and pre-designed siRNA targeting HO-1 (HO-1-siRNA) to silence the human HO-1 gene were purchased from Ambion (Foster City, CA, USA). For transfection, cells were seeded into 6-well plates, incubated for 24 h, and then transfected according to the manufacturer's instructions. After subsequent treatments, cells were harvested for flow cytometry and for protein extraction for use in western blot experiments.

Real-time PCR

Human NP samples from 6 IDD patients and 6 LVF patients were selected randomly for real-time RT-PCR analysis. Total RNA was extracted from human NP tissues and cells using TRIzol Reagent (Invitrogen, 15596-018) by using RNAiso PLUS (Takara, Japan) according to the manufacturer's instructions. Next, the RNA samples were used to synthesize cDNA using the PrimeScript[®] RT reagent Kit with gDNA Eraser (Takara, RR047Q). Relative expression levels of the indicated genes were calculated using the 2- $\Delta\Delta C_t$ method. Primers for the amplification of genes encoding HO-1, NF-κB P65 and GAPDH were synthesized by Takara (TaKaRa, China). Primers for human genes were designed as follows:

5'-AGTCTTCGCCCTGTCTACT-3' and 5'-GCTGGTGTGTAGGGGATGAC-3' for HO-1;

5'-ACAACCCCTTCCAAGTTCCT-3' and 5'-TGGTCCCGTGAAATACACCT-3' for NF-κB P65;

5'-CAATGACCCCTTCATTGACC-3' and 5'-GACAAGCTTCCCGTTCTCAG-3' for GAPDH.

Western blot analysis

Human NP samples from 6 IDD patients and 6 LVF patients were selected randomly for western blot analysis. The tissues were homogenized, and the cells were lysed on ice with RIPA Lysis Buffer (Beyotime, China). Protein concentrations were determined using an Enhanced BCA Protein assay kit (Beyotime, P0010S). Samples containing 50 µg of proteins were electrophoresed in 6–12% SDS-PAGE gels, and proteins were transferred onto PVDF membranes, which were blocked with 5% nonfat dry milk in Tris-buffered saline (TBST) for 1 h. Then, membranes were incubated with primary antibodies (rabbit anti-HO-1 (#5853), rabbit anti-P65 (#8242), rabbit anti-p-P65 (#3033), rabbit anti-cleaved caspase 3 (#9661), rabbit anti-Bax (#5023), rabbit anti-Bcl-2 (#3498), and mouse anti-β-actin (Beyotime Institute of Biotechnology; cat. no. AF0003)) overnight at 4°C. The membranes were washed three times with TBST for 15 min and incubated in secondary antibody at 37°C for 1 h. Finally, the membranes were visualized using ECL Plus Reagent (Beyotime, China), and the results were analysed by the software.

Immunofluorescence Staining

The cells prepared were fixed with 4% paraformaldehyde for 10 min and treated with 5% Triton for 5 min. Then, the cells were stained with the rabbit anti-P65 antibody (1:100) at 4°C overnight. Next, the cells were incubated in secondary antibody at 37°C for 1.5 h. Finally, the coverslips were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and imaged using a confocal laser scanning microscope (Olympus).

Statistical analysis

The experiments were performed at least three times. The results are presented as the mean±standard deviation (SD). Statistical analyses were performed using SPSS 17 statistical software (SPSS Inc., IL, USA). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparisons between two groups; $p < 0.05$ was considered statistically significant.

Results

Less expression of HO-1 in NP from IDD patients than in NP of LVF patients

Immunohistochemistry of human NP tissues showed that HO-1 and P65 were expressed in all tissues. Compared with the LVF group, the IDD group showed decreased numbers of HO-1-positive cells and significantly increased numbers of P65-positive cells (Fig. 2A, B).

Apoptosis of NP specimens from the LVF and IDD groups was measured by TUNEL labelling. The results indicated that there were more TUNEL-labelled apoptotic cells in the IDD group than in the LVF group (Fig. 2C).

Real-time PCR results showed that the mRNA expression levels of HO-1 of NP from the IDD group were lower than those of NP from the LVF group. In contrast, the mRNA expression level of P65 was higher in NP from the IDD group than in NP from the LVF group. Similarly, the western blot results showed that compared with the LVF group, NP samples from the IDD group showed lower expression of HO-1 and higher expression of P65. (Fig. 2D, E).

IL-1β increased P65 phosphorylation and nucleus pulposus cell apoptosis

To demonstrate the relationship between NF-κB and apoptosis, degenerative human NPCs were treated with IL-1β (10 ng/mL) for 24 h. Western blot results show that after treatment with IL-1β, the expression levels of Bax, cleaved caspase 3 and phosphorylated P65 (p-P65) were significantly up-regulated, and the expression of the anti-apoptosis protein Bcl-2 was markedly down-regulated (Fig. 3A). Simultaneously, the results of Annexin V/PI double staining also demonstrated that the apoptotic rate of human NPCs was obviously increased after treatment with IL-1β (Fig. 3B).

HO-1 decreased apoptosis and inhibited the NF-κB pathway in human NPCs treated with IL-1β

To understand whether HO-1 can protect degenerative human NPCs against apoptosis and to reveal its possible mechanism, HO-1 expression in NPCs was increased by using LV-

Fig. 2. The expression of HO-1, P65 and apoptosis in NP tissues from the LVF and IDD groups. (A) Immunohistochemistry results show that HO-1 levels are decreased in NPs from patients with IDD (n=3, 200 \times , *P<0.05); (B) P65 levels are increased in NPs from patients with IDD (n=3, 200 \times , *P<0.05). (C) The number of apoptotic cells was determined by TUNEL staining in NP tissues, and the results show that the percentages of apoptotic cells are elevated in NP tissues from patients with IDD (n=3, 200 \times , *P<0.05). (D) Western blotting results show decreased expression of HO-1 protein and increased expression of P65 protein in NP tissues from patients with IDD (*P<0.05); (E) Real-time PCR results show decreased mRNA expression of HO-1 and increased mRNA expression of P65 in NP tissues from patients with IDD (*P<0.05).

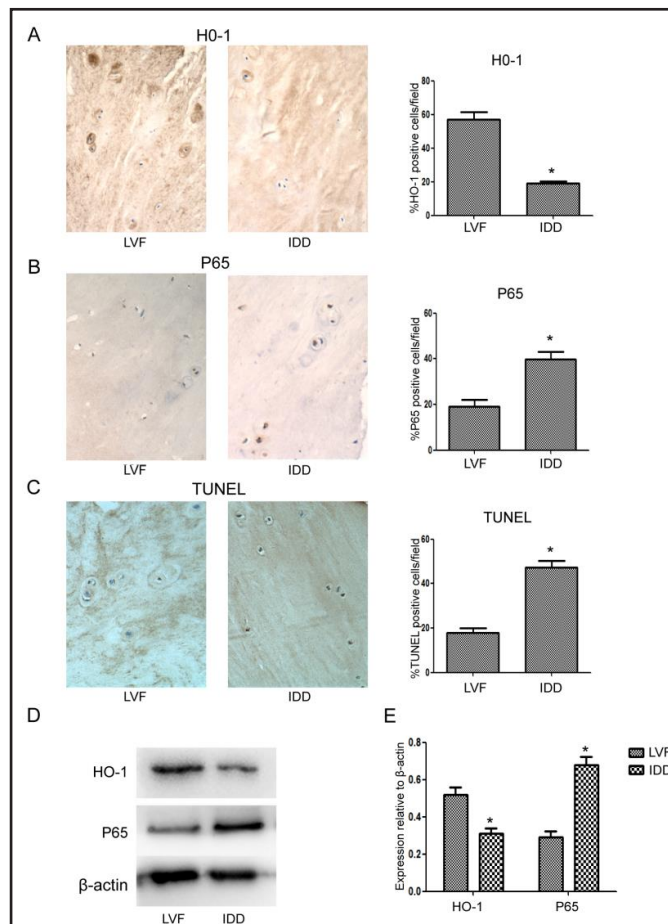


Fig. 3. IL-1 β increases P65 phosphorylation and nucleus pulposus cell apoptosis. (A) Western blot results showed that the expression levels of Bax, cleaved caspase 3 and p-P65 were significantly up-regulated, and the expression of the anti-apoptotic protein Bcl-2 was markedly down-regulated after treatment with IL-1 β . (B) Flow cytometry revealed that the apoptotic rate of human NPCs was markedly increased after treatment with IL-1 β . Data are presented as the mean \pm SD; *P<0.05 compared with the control.

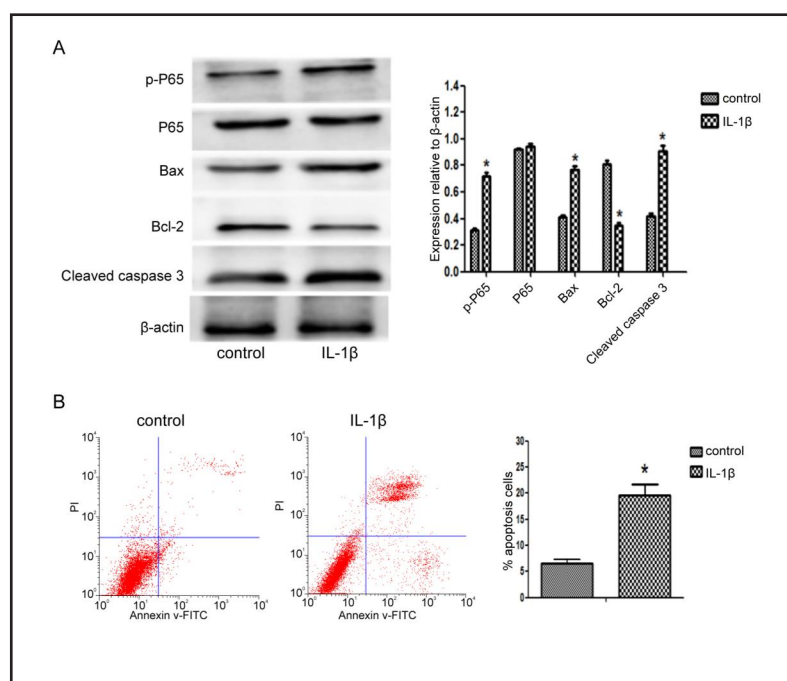
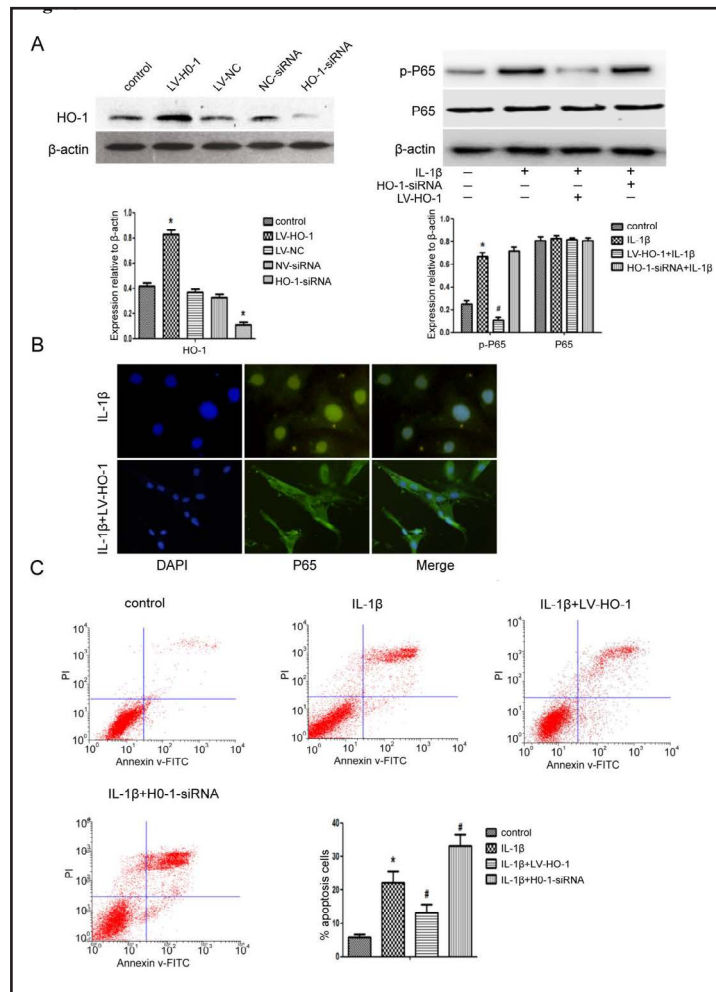


Fig. 4. HO-1 decreases apoptosis and inhibits the NF- κ B pathway in human NPCs treated with IL-1 β . (A) Western blot results showed that compared with the control group, the level of HO-1 protein expression was increased after treatment with LV-HO-1 and was reduced after transfection with siRNA targeting HO-1. Western blot results also revealed that the protein expression of p-P65 was significantly up-regulated after treatment with IL-1 β . However, after the human NPCs were treated with LV-HO-1, the protein expression of p-P65 was significantly down-regulated. The protein expression level of p-P65 did not change after treatment with HO-1-siRNA. (B) Immunofluorescent assay results showed that P65 was mainly located in the nucleus after treatment with IL-1 β alone and translocated into the cytoplasm when LV-HO-1 was added. (C) The incidence of apoptosis in degenerative human NP cells was evaluated by flow cytometry. The incidence of apoptosis was significantly increased after IL-1 β treatment. Moreover, the incidence of apoptosis was increased more obviously in HO-1-siRNA-treated cells than in IL-1 β -treated cells. However, the incidence of apoptosis was decreased in LV-HO-1-treated cells. The data are presented as the mean \pm SD; * P <0.05 compared with the control, # P <0.05 compared with the IL-1 β group.

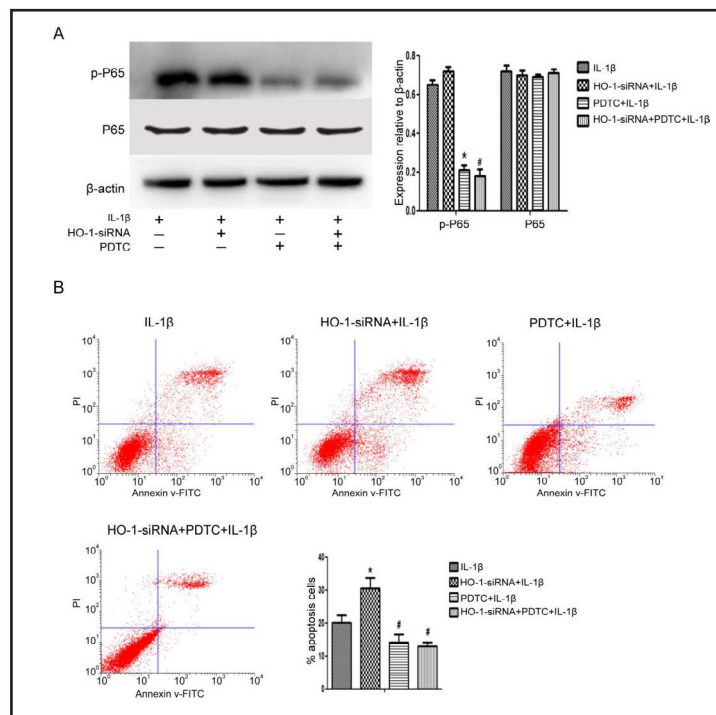


HO-1 and decreased by transfection with HO-1-siRNA. Western blot results showed that compared with the control group, the level of HO-1 protein expression was increased after treatment with LV-HO-1 and decreased after transfection with siRNA targeting HO-1 (Fig. 4A).

Western blot results also revealed that the protein expression of p-P65 was significantly up-regulated after treatment with IL-1 β . However, after the human NPCs were treated with LV-HO-1, the protein expression of p-P65 was significantly down-regulated. The protein expression level of p-P65 did not change after treatment with HO-1-siRNA. These results indicated that HO-1 inhibited the IL-1 β -induced activation of the NF- κ B pathway in human NPCs (Fig. 4A).

To understand how HO-1 affects P65, an immunofluorescent assay was used to test changes in the the P65 localization pattern after treatment with IL-1 β or IL-1 β +LV-HO-1. We found that P65 was mainly located in the nucleus after treatment with IL-1 β alone and was translocated into the cytoplasm when LV-HO-1 was added (Fig. 4B). The results show that HO-1 may inhibit NF- κ B by inhibiting P65 nuclear translocation in degenerative human NPCs.

Fig. 5. The inhibition of p-P65 prevented IL-1 β -induced apoptosis with or without HO-1-siRNA. (A) Western blot results showed that the levels of p-P65 were markedly increased in NPCs stimulated with IL-1 β compared with the control cells. However, when NPCs were treated with 10 μ M PDTC for 24 h, the level of p-P65 was markedly decreased in NPCs treated with or without HO-1-siRNA. (B) The percentage of apoptotic cells was measured by flow cytometry. The incidence of apoptosis increased markedly after HO-1-siRNA treatment. However, the incidence of apoptosis markedly decreased in PDTC-treated cells. The data are presented as the mean \pm SD; * P <0.05 compared with the control, # P < 0.05 compared with the IL-1 β group.



To confirm the role of HO-1 in the regulation of apoptosis of human NP cells, we performed flow cytometric analysis using Annexin V/PI. Flow cytometric analysis showed that the incidence of apoptosis was significantly increased after IL-1 β treatment. Moreover, the incidence of apoptosis was increased more obviously in HO-1-siRNA-treated NPCs compared with that in IL-1 β -treated NPCs. However, the incidence of apoptosis was decreased in cells treated with LV-HO-1 (Fig. 4C). These results revealed that HO-1 could inhibit IL-1 β -induced apoptosis in NPCs.

The inhibition of p-P65 prevented IL-1 β -induced apoptosis with or without HO-1-siRNA

The NF- κ B signalling pathway has previously been reported to regulate cell survival and apoptosis [16]. To further confirm whether HO-1 can protect degenerative human NPCs against apoptosis through the NF- κ B pathway, human NPCs were treated with the NF- κ B inhibitor PDTC or with HO-1-siRNA. Western blot results showed that the level of p-P65 was markedly increased in NPCs stimulated with IL-1 β compared with the control cells. However, when NPCs were treated with 10 μ M PDTC for 24 h, the level of p-P65 in NPCs was markedly decreased, with or without treatment with HO-1-siRNA (Fig. 5A). Furthermore, Annexin V/PI apoptosis detection analysis demonstrated that the incidence of apoptosis was markedly increased in HO-1-siRNA-treated NPCs compared with that in IL-1 β -treated cells. However, the incidence of apoptosis was significantly decreased in

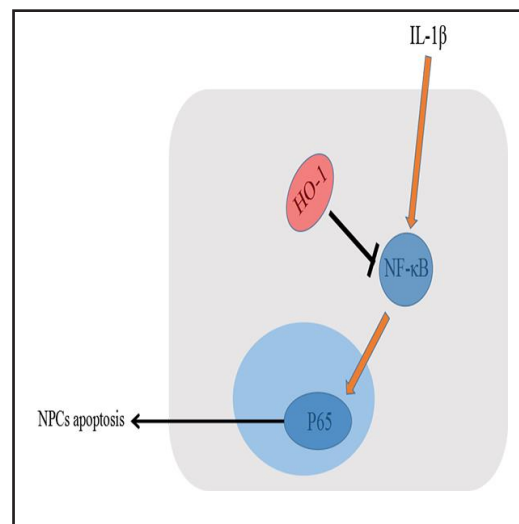


Fig. 6. Schematic diagram: the effect and mechanism of HO-1 on apoptosis in human degenerative NPCs.

PDTC-treated cells whether or not HO-1-siRNA was added (Fig. 5B). These results indicated that the anti-apoptotic effect of HO-1 on human NPCs was mediated via the NF- κ B signalling pathway (Fig. 6).

Discussion

In the present study, we confirmed for the first time that HO-1 protects against IL-1 β -induced apoptosis in human degenerative NPCs. We observed that human degenerative IVD nucleus pulposus tissues showed down-regulated expression of HO-1, up-regulated expression of P65, and increased apoptosis, and these results were consistent with previous studies [9, 16, 17]. Thus, we hypothesized that HO-1 and NF- κ B may play a significant role in the process of NPC apoptosis. To test this hypothesis, human degenerative NPCs were treated with HO-1-siRNA or LV-HO-1 in the presence of IL-1 β . The results demonstrated that the number of apoptotic NPCs was significantly decreased following overexpression of HO-1, which simultaneously led to inhibition of the NF- κ B pathway. Furthermore, the NF- κ B inhibitor PDTC was used to treat NPCs, and the number of apoptotic NPCs was significantly decreased, which suggested that the anti-apoptotic effects of HO-1 were mediated via the NF- κ B signalling pathway.

It is well established that the excessive apoptosis of NPCs plays an important role in the process of IDD. An IVD consists of nucleus pulposus (NP), annulus fibrosus (AF) and cartilage end plates (CEPs). The low number of NP cells caused by excessive apoptosis leads to a reduction in the synthesis of ECM. Finally, excessive reduction of the ECM results in IVD degeneration. A previous study demonstrated that the number of apoptotic cells and the level of the apoptotic protein caspase-3 in degenerative NP specimens were markedly increased compared with the normal control [18, 19]. Ahsan et al. [20] also showed that in degenerative disc samples, the levels of apoptosis were higher. In our study, we observed that the number of apoptotic cells in the IDD group was greater than that in the LVF group. Simultaneously, Bo Hu et al. [9] indicated that the expression levels of HO-1 in NP from severe IDD were lower than in those of NP from mild IDD. In our study, the numbers of HO-1-positive cells in the IDD group were significantly decreased compared with the LVF group. Furthermore, the mRNA and protein expression levels of HO-1 in the IDD group were also decreased compared with the LVF group. However, to our knowledge, the effect of HO-1 on the apoptosis of degenerative human NPCs has never been reported. Therefore, we hypothesize that HO-1 participates in the process of apoptosis in intervertebral disc NPCs.

Hemeoxygenase acts as the rate-limiting enzyme in the degradation of haeme [21, 22], and it has two functionally active isoforms, HO-1 and HO-2. HO-1 predominantly catabolizes intracellular haeme and convert it to bilirubin, carbon monoxide (CO), and free iron [23]. Many studies have reported that HO-1 plays an important role in apoptosis and ageing. For example, overexpression of HO-1 significantly decreased the number of apoptotic cells induced by high glucose in H9c2 cells [24, 25]. Moreover, upregulating hemeoxygenase-1 expression by curcumin inhibits anthracycline-induced apoptosis in SHSY5Y cells [26]. Thus, we hypothesized that HO-1 could protect degenerative human NP cells against apoptosis. In this study, LV-HO-1 and HO-1-siRNA were used to treat human degenerative NPCs in the presence of IL-1 β . The results showed that the incidence of apoptosis in NPCs was significantly decreased in the group in which HO-1 was activated by LV-HO-1 compared with the IL-1 β group. These observations clearly indicated that HO-1 could protect against IL-1 β -induced apoptosis of degenerative human NPCs. There was an interesting result in this part of the experiment: we found that the level of p-P65 was slightly higher in the presence of IL-1 β after treatment with HO-1-siRNA than in the presence of IL-1 β alone. However, this difference is small and nonsignificant. Nonetheless, the NP cells in the HO-1-siRNA treatment group had a higher apoptosis rate. We consider that the level of p-P65 is elevated to a fairly high level (perhaps near the peak). Therefore, treatment with HO-1-siRNA did not significantly increase the level of p-P65. However, the change in the apoptosis rate, as a result

of signal pathway regulation, may be magnified due to the “cascade effect” of the signalling pathway.

Nuclear factor kappa B (NF- κ B) is a transcription factor that plays an important role in the response of the cell to injury, stress, apoptosis and inflammatory reactions [27]. The NF- κ B signalling pathway is significantly correlated with the process of IDD. Inhibiting the activation of NF- κ B can significantly retard the degree of disc degeneration [13, 28]. It was also reported that the SDF-1/CXCR4 axis induces apoptosis of human degenerative NPCs via the NF- κ B pathway [29]. Our results also indicated that the levels of apoptosis and P65 in degenerated intervertebral disc tissue were significantly higher than in normal intervertebral disc tissue, suggesting that the NF- κ B signalling pathway was involved in NPC apoptosis. However, whether the anti-apoptotic effect of HO-1 in intervertebral disc NPCs occurs through the NF- κ B signalling pathway is not clear. Our research demonstrated that treatment with the NF- κ B inhibitor PDTC inhibited the number of apoptotic NPCs and suppressed P65 phosphorylation and that the overexpression of HO-1 could not rescue the apoptosis caused by NF- κ B pathway inhibition. This indicated that the anti-apoptotic effects of HO-1 were mediated via the NF- κ B signalling pathway.

However, our study has some limitations. Regarding sample collection, it is difficult to obtain completely healthy NP tissues in clinical work. Therefore, we chose the relatively normal NP tissues obtained from young patients with LVF as normal controls. Nevertheless, relatively normal human intervertebral disc tissues are also difficult to obtain in clinical work, so we obtained only 6 intervertebral discs from LVF patients.

In conclusion, all the results indicated that HO-1 is an important mediator of apoptosis in degenerative human disc NPCs. HO-1 can regulate the apoptosis of NPCs through the NF- κ B pathway. Therefore, HO-1 may become a novel potential therapeutic approach for IDD treatment.

Disclosure Statement

No conflicts of interests exist.

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