GSK-3β Inhibitor SB216763 Resists Oxidative Stress-Induced Apoptosis in Nucleus Pulposus Cell

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Abstract

Oxidative stress in the intervertebral disc leads to nucleus pulposus (NP) degeneration by inducing cell apoptosis. However, the molecular mechanisms underlying this process remain unclear. Increasing evidence indicates that GSK-3β is related to cell apoptosis induced by oxidative stress. In this study, we explored whether GSK-3β inhibition protects human NP cell against apoptosis under oxidative stress. Immunofluorescence staining was used to show the expression of GSK-3β in human NP cells (NPCs). Flow cytometry, mitochondrial staining and western blot were used to detect apoptosis of treated NPCs, changes of mitochondrial membrane potential and the expression of mitochondrial apoptosis-related proteins using GSK-3β specific inhibitor SB216763. Coprecipitation was used to demonstrate the interaction between GSK-3β and Bcl-2 in an GSK-3β knockdown in vitro model. We delineated the protective effect of GSK-3β specific inhibitor SB216763 on human NP cell apoptosis induced by oxidative stress in vitro. Further, we showed SB216763 exert the protective effect by preservation of the mitochondrial membrane potential and inhibition of caspase 3/7 activity during oxidative injury. The detailed mechanism underlying the antiapoptotic effect of GSK-3β inhibition was also studied by analyzing mitochondrial apoptosis pathway in vitro. We concluded that the GSK-3β inhibitor SB216763 protected mitochondrial membrane potential to delay nucleus pulposus cell apoptosis by inhibiting the interaction between GSK-3β and Bcl-2 and subsequently reducing Cyto-C release and caspase-3 activation. Together, inhibition of GSK-3β using SB216763 in NP may be a favorable therapeutic strategy to slow intervertebral disc degeneration.

Introduction

Intervertebral disc degeneration (IVDD) is a major cause of chronic lower back pain, imposing a heavy burden on individuals, especially in elderly people [1]. Lumbar fusion surgeries remain the mainstay of therapy for patients who are refractory to conservative treatments. However, such surgeries can result in impaired mobility of the lumbar spine, followed by accelerated degeneration of the adjacent segments [2]. In the past few decades, researchers have made great efforts to delay the degeneration of lumbar intervertebral discs by undertaking in-depth study of IVDD at the molecular level. The intervertebral disc is structurally composed of cartilage end plates (CEPs), annulus fibrosus (AF) and nucleus pulposus (NP). Studies have shown that IVDD originates in NP degeneration and is characterized by a significant decrease in chondrocytes, proteoglycans and type II collagen [3, 4]. The change in the NP microenvironment leads to increased apoptosis of nucleus pulposus cells and a reduction in matrix synthesis. Therefore, NP apoptosis has become a promising therapeutic target for IVDD.

Nucleus pulposus is a hypoxic tissue due to the lack of blood supply [5]. NP nutrition mainly comes from the cartilage endplate and the outer annulus fibrosus. The hypoxic microenvironment is vital for the survival of NP cells [6]. After AF injury, blood vessels grow into the NP and disrupt the hypoxic microenvironment, leading to oxidative stress in the NP [7]. The literature has shown that ROS are increased in human degenerative NP compared with normal NP, which is accompanied by NP cell apoptosis [2, 8–10]. In recent years, growing evidence suggests that GSK-3β is intimately associated with

cell apoptosis induced by oxidative stress [11–13]. Juhaszova et al. found that inhibition of GSK-3β activity can enhance the tolerance of cardiomyocytes to oxidative stress injury [14]. In a study of IVDD, Risbud et al. demonstrated that GSK-3β inhibition can ameliorate the apoptosis of rat NP cells by activating the PI3K/Akt pathway and MAPK pathway [15]; however, a study by Hiyama et al. showed that GSK-3β inhibition can aggravate NP cell apoptosis by activating the Wnt pathway and upregulating TNF-α expression [16]. The inconsistency of these conclusions reflects the complexity of the mechanisms by which GSK-3β regulates the apoptosis of NP cells.

SB216763 is a selective GSK-3β inhibitor with a small molecular weight and has been widely used in GSK-3β related studies [17]. SB216763 has been shown to protect against apoptosis of cardiomyocytes [18], nerve cells [19], hepatocytes [20] and crystalline epithelial cells [21]. However, its effect on NP apoptosis has not been reported in the literature. In the present study, we aimed to explore the effects of SB216763 and GSK-3β inhibition on human NP apoptosis induced by oxidative stress and to determine the underlying mechanisms.

Materials And Methods

Ethical approval

The collection of human NP samples was approved by the Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine. All experiments were conducted in accordance with the Helsinki Declaration. All patients and the next of kin were fully legally competent and consented to the use of lumbar NP tissues for research. Written informed consent from the patients or the next of kin was obtained. None of the patients belonged to a vulnerable population, and all patients or next of kin freely provided written informed consent. The privacy rights of the patients or next of kin were always protected. The patients or the next of kin provided written informed consent to publish case details in this manuscript.

Extraction, culture and identification of NPCs

NP samples in percutaneous endoscopic lumbar discectomy (PELD) surgery were collected, 0.25% trypsin was added, and the samples were placed in a 37° incubator for digestion for half an hour. After centrifugation at 1500 RPM/min, the supernatant was removed, 0.1% type II collagenase was added (diluted with 10% FBS), and the cells were collected in an incubator at 37 °C overnight. Cultured cells were validated by immunofluorescence staining with aggrecan and collagen-II antibodies (Cell Signaling), which are NP cell-specific markers.

Immunofluorescence staining in vitro

Twenty thousand living cells were loaded onto each coverslip and incubated overnight. PFA (4%) was fixed at room temperature for 15 min. Then, 1 mL of blocking solution (containing 10% horse serum, 1% BSA and 0.1% Triton X-100 DPBS) was added and blocked for 1 hour. The blocking solution was

discarded, and diluted primary antibodies (including Aggrecan, Collagen-II, GSK-3β, Caspase-3/7, and 8-OHDG) were added for incubation. After rinsing, the coverslips were placed in the dark and incubated in florescent-labeled goat-anti-rabbit IgG for 1 hour. Then, Prolong Gold antifade reagent with DAPI was added to the coverslips. All coverslips were randomly photographed, and at least 200 cells were counted (Leica DMI 6000B).

Flow cytometry

NP cells were stimulated with different concentrations of TBHP (Sigma–Aldrich) for 6 h, 12 h and 24 h. The cells were collected and treated with the pre-prepared 1× Annexin V Binding Solution at a final concentration of 5×10^5 cells/mL. Annexin V-APC (5 ml) and PI (5 ml) were added, and NPCs were cultured at room temperature in the dark for 15 min. Apoptosis was detected by flow cytometry within 1 h. Similarly, NPCs were treated with 175 μM TBHP for 12 h and then precipitated and resuspended in 37° preheated TMRM staining fluid for 30 min. The 488 nm laser excitation and 570±10 nm emission filter were used to detect flow data and further analyzed by FlowJo software (7.6.1).

TBHP cytotoxicity assay (CCK8 method

NPCs in logarithmic growth phase were made into a cell suspension. NPCs were loaded in a 96-well plate and cultured at 37 °C in a 5% CO2 incubator. NPCs were treated with 100 μl of TBHP at different concentrations for 12 h. Ten microliters of CCK8 solution was added to a 96-well plate, and the absorbance at 450 nm was measured.

Mitochondrial staining

TMRM is a commonly used fluorescent dye that depends on mitochondrial membrane potential, and MTG is a fluorescent dye that is independent of mitochondrial membrane potential. Both of them can stain the mitochondria of living cells. When NPCs grew to 80% confluence, they were treated with 175 μM TBHP for 12 h, and then TMRM and MTG staining working solutions were added for incubation. After rinsing, DAPI (5 μg/ml) was used to stain nuclei. Photographs were taken with a confocal microscope.

Western blot

The attached NP cells were trypsinized and lysed using RIPA (Bio-Rad) to extract the total proteins. Samples were denatured and loaded into sodium dodecyl sulfate-polyacrylamide gels to run for 1 hour at 100 V. The proteins were transferred to a polyvinylidene difluoride membrane (Bio–Rad). After blocking with 5% milk solution, the membranes were incubated in primary antibody solution overnight at 4 °C. The membranes were incubated with the appropriate diluted HRP-conjugated secondary antibody (1/2000 in 1× TBST) for 1 hour at room temperature. ECL chemical luminescent agents were prepared and exposed and developed with a chemiluminescence imager.

GSK-3β knockdown in NPCs

The lentivirus stock was diluted with fresh medium containing 5 μg/mL polycoagulant. The dilution containing negative control (NC) was added to the control group, and the dilution containing lentivirus was added to the treatment group. The culture medium containing lentivirus was replaced with normal culture medium 16 h after infection. Western blot and qRT–PCR were used to detect the effectiveness of lentivirus infection.

Extraction of RNA and quantitative reverse transcription-polymerase chain reaction (qRT–PCR)

Total RNA of NPCs was extracted and purified using a QIAGEN mini kit. After that, one microgram of RNA was reverse transcribed, and complementary DNA was subjected to PCR. GAPDH was used as the normalizing gene. The primers used were as follows:

GSK-3β Forward CCCAGAACCACCTCCTTTGC

GSK-3β Reverse TGCCACCACTGTTGTCACCTT

GAPDH Forward GTCTCCTCTGACTTCAACAGCG

GAPDH Reverse ACCACCCTGTTGCTGTAGCCAA

The mRNA expression level of GSK-3β was measured and quantified using an ABI Prism 7000 sequence detection system.

Coprecipitation (CO-IP)

The cells were collected with a scraper, and the supernatant was assembled after centrifugation. Two hundred microliters was taken for the input sample, 400 μl for the IgG sample, and 400 μl for the co-IP sample. Input samples were added to 5× SDS–PAGE protein loading buffer and placed at -20 °C for later use. The IgG and co-IP samples were separately added with a primary antibody for immunoprecipitation (dilution ratio 1:50). Then, 50 μl of pretreated magnetic beads was added, mixed and incubated at 4° for 3 h. Sixty microliters of 5×SDS–PAGE protein loading buffer was added. The IP samples were boiled in boiling water for 10 mins. After cooling, the protein samples were loaded for verification using western blotting.

Statistical analysis

All data are expressed as the mean±SD. Statistical analyses were performed by using SPSS (version 23.0, SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using one-way analysis of variance and independent samples t-test. Post hoc pairwise comparisons were performed using Dunnett's T3 or Student-Newman–Keuls (S-N-K) test. A value of $P<0.05$ was considered statistically significant.

Results

Expression of GSK-3β in NPCs

We successfully cultured nucleus pulposus cells in vitro, and immunofluorescence showed positive coexpression of Aggrecan and Collagen II in the cultured cells (Fig. S1). Gsk-3 β was expressed in NPCs, mainly in the cytoplasm, with a small amount of expression in the nucleus (Fig. S2).

TBHP induced NPC apoptosis

In the 6 h treatment condition, different concentrations of TBHP did not lead to a significant increase in the cell apoptosis rate compared with the control group, while in the 24 h treatment condition, the cell necrosis rate significantly increased (Fig. 1a). Under the 12 h treatment condition, when the intervention concentrations were 150 and 175 μM, the cell necrosis rate was similar, while the apoptosis rate was significantly different (Fig. 2b-d). Therefore, the appropriate treatment condition for TBHP was 175 μM for 12 h. In addition, we further examined the effects of TBHP on cytotoxicity and oxidative stress in the nucleus pulposus (Fig. S3 and Fig. S4).

SB216763 ameliorated NPC apoptosis

We verified the effect of SB216763 on NP cell apoptosis induced by TBHP. The results showed that the cell apoptosis rate significantly decreased from 32.20±1.35% to 20.73±1.45% after SB216763 treatment (Fig. 2a-b).

SB216763 protected the loss of mitochondrial membrane potential induced by TBHP

Flow cytometry was used to detect TMRM fluorescence intensity. The results showed that TBHP caused a decrease in TMRM fluorescence intensity, which indicates the disruption of mitochondrial membrane potential. After SB216763 treatment, the TMRM fluorescence intensity was increased compared with that of the TBHP group, suggesting that the mitochondrial membrane potential was maintained (Fig. 3a, Fig. S5). We further analyzed the fluorescence intensity of TMRM with laser confocal microscopy and confirmed that SB216763 protected against the loss of mitochondrial membrane potential induced by TBHP (Fig. 3b-c).

SB216763 exerted protective effects through preservation of Bcl-2 expression,

followed by the suppression of cytochrome c release and caspase-3 activation

The results of immunofluorescence detection showed that TBHP increased the fluorescence intensity of caspase 3/7, which indicated enhanced caspase 3/7 activity. After SB216763 treatment, the fluorescence intensity of caspase 3/7 decreased (Fig. 4a). WB results showed that TBHP exposure facilitated cytochrome c release in the cytosol, decreased Bcl-2 expression, and increased the expression of cleaved caspase-3, all of which could be suppressed by SB216773 (Fig. 4b) (Fig. 4c). We further analyzed the effect of SB216763 on the phosphorylation of Bcl-2 at Ser70 using western blotting. TBHP induced the increased expression of phosphorylated Bcl-2 at Ser70. After administration of SB216763, its expression decreased (Fig. 4d).

Knocking down GSK-3β protects mitochondrial apoptosis-related proteins that were induced by TBHP

We successfully constructed an in vitro GSK-3β knockdown model using lentivirus infection and verified it by q-RTPCR and western blot (Fig. S6). We used western blotting to detect changes in mitochondrial apoptosis-related proteins after GSK-3β knockdown in vitro. The results showed that TBHP induced a decrease in Bcl-2 expression and an increase in cleaved caspase-3 expression in NPCs, while knocking down GSK-3β gene expression inhibited the TBHP-induced decrease in Bcl-2 expression and the increase in cleaved caspase 3 (Fig. 5a-b). The results of coimmunoprecipitation showed (Fig. 5c) that GSK-3β protein was also precipitated when the Bcl-2 antibody was used to precipitate the Bcl-2 protein, which indicated that Bcl-2 interacted with GSK-3β.

Schematic representation illustrating the role of SB216763 in intervertebral discs.

Overall, we clearly delineated that the GSK-3β inhibitor SB216763 protected mitochondrial membrane potential to delay nucleus pulposus cell apoptosis by inhibiting the interaction between GSK-3β and Bcl-2 and subsequently reducing Cyto-C release and caspase-3 activation (Fig. 5d).

Discussion

In this study, we successfully established an in vitro oxidative stress injury

model of human NP cells. Double-labeled immunofluorescence was used to detect the expression of Aggrecan and Collagen Ⅱ, which are two common markers of NP cells [22]. The results indicated that GSK-3β was widely expressed in human NP cells. In recent years, an increasing number of studies have shown that GSK-3β is widely involved in cell oxidative stress and apoptosis [14, 23-25]. However, there remains controversy about the role of GSK-3β in NP apoptosis. Risbud et al. showed that inhibition of GSK-3β ameliorated apoptosis of rat NPCs by activating the PI3K/Akt pathway and MAPK pathway [15]. However, Hiyama et al. showed that inhibition of GSK-3β increased the expression of TNF-α and aggravated the apoptosis of NPCs by activating the Wnt pathway [16]. We found that GSK-3β inhibition by SB216763 or by shRNA could protect NP cells against oxidative stress injury through a mitochondrial mechanism.

Mechanisms of cell apoptosis can be summarized into two categories:

exogenous and endogenous. The exogenous pathway is characterized by the transmission of apoptotic signals through death receptors located on the cell surface. The endogenous pathway involves the activation of caspase-3, followed by a loss of mitochondrial membrane potential (ΔΨm). ΔΨm is an electrochemical gradient generated by proton pumps across the mitochondrial inner membrane [26, 27]. It is regulated by the opening status of the mitochondrial permeability transition pore (mPTP) [28]. ΔΨm damage or opening of the mPTP is considered the initiation step of mitochondrial apoptosis. After that, cytochrome C (Cyto-C) is released from the mitochondrial space into the cytoplasm, which then forms an apoptotic complex with apoptosis-initiating proteins, eventually inducing cell apoptosis [29]. First, we

explored whether SB216763 can protect ΔΨm. Our results showed that SB216763 helped to preserve the mitochondrial membrane potential during oxidative injury. Next, we examined the effects of SB216763 on the activation of caspase 3 and found that the expression level of caspase 3/7 was decreased after intervention with SB216763, indicating that the activity of caspase 3/7 was inhibited.

Bcl-2 family proteins have been recognized as key regulators involved in mitochondrial apoptosis. Our results showed that the expression of antiapoptotic Bcl-2 protein was decreased and that proapoptotic proteins, including Cyto-C and C-caspase 3, were increased during oxidative injury triggered by TBHP. These changes in protein levels can be reversed by SB216763. Hence, we believe that SB216763 exerts protective effects by regulating the expression of Bcl-2 family proteins. It was reported that the role of Bcl-2 in oxidative injury was associated with the phosphorylation status of the serine-70 site [30]; therefore, we analyzed the changes in S70-phosphorylated Bcl-2 at the protein level. WB results showed that SB216763 prevented the increase in phosphorylated BCL-2 protein induced by TBHP. Previous studies have demonstrated that phosphorylation of BCL-2 at Ser70 could lead to Bcl-2 degradation [31, 32]. Hence, we hypothesized that phosphorylation of Bcl-2 at S70 impaired the antiapoptotic effect of Bcl-2. In summary, SB216763 ameliorated NPC apoptosis by protecting ΔΨm and inhibiting the release of Cyto-C and activation of caspase 3, which were probably mediated by the preservation of Bcl-2 protein levels.

To further study the mechanisms of the antiapoptotic effect of GSK-3β inhibition. We constructed a stable NP cell line with GSK-3β knockdown in vitro using shRNA. The results revealed that knockdown of GSK-3β could suppress the decrease in Bcl-2 protein expression and the increase in cleaved caspase 3 protein expression induced by TBHP. This was consistent with previous findings that indicated that GSK-3β is an upstream regulator of Bcl-2 [33]. A previous study also proved that Bcl-2 can be phosphorylated by GSK-3β [32], so we further conducted coimmunoprecipitation (Co-IP) to determine the interaction between Bcl-2 and GSK-3β. Our results confirmed that Bcl-2 was a substrate of GSK-3β.

This study has certain limitations that should be discussed. First, the exact role of BCL-2 phosphorylation at different sites in NPC apoptosis remains ambiguous. To verify this, the restriction site mutation technique should be used in our future work. Second, there is a lack of in vivo experiments. In conclusion, GSK-3β inhibition protected human NP cells against oxidative stress-induced apoptosis by stabilizing Bcl-2 levels, which was followed by the suppression of cytochrome c release and caspase-3 activation. This effect might be mediated by the interaction between GSK-3β and Bcl-2.

Declarations

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Data availability: All data generated or analyzed during this study are included in this published article and available from the corresponding author upon reasonable request.

Competing Interests:

The authors declare no conflict of interest.

Authors Contributions:

Kai Zhu: acquisition of data, Drafting the manuscript

Song Guo: Drafting the manuscript, Validation and Methodology

Guoyi Han: Analysis and/or interpretation of data

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Zhijun Zhang: Validation and Methodology

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Shaopeng Li: Review the manuscript

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All authors approved to submit this version to this publication.

Ethics approval:

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent to publish: The authors affirm that human research participants provided informed consent for publication of the images.

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Supplementary Figures

Supplementary Figures S1-S6 are not available with this version

Figures

Figure 1

TBHP induced NPCs apoptosis. a Flow cytometric analysis of apoptosis with different concentrations of TBHP for 6h. **b** Flow cytometric analysis of apoptosis with different concentrations of TBHP for 12h. **c** Flow cytometric analysis of apoptosis with different concentrations of TBHP for 24h. **d** The apoptosis and necrosis rates of treated NPCs at different time points.

Figure 2

SB216763 improved apoptosis of treated NPCs. a Flow cytometric analysis of apoptosis in each treatment group as described above. **b** Apoptosis rate in each treatment group as described above. Data are expressed as the mean ± SD.

Figure 3

SB216763 protected mitochondrial membrane potential in treated NPCs. a Flow cytometric analysis of mitochondrial membrane potential in each treatment group as described above. **b** Mean fluorescent intensity (MFI) values of laser confocal in each treatment group as described above. Data are expressed

as the mean ± SD. **c** Laser confocal analysis of mitochondrial membrane potential in each treatment group as described above.

Figure 4

SB21676 protected the mitochondrial apoptosis-related proteins that were induced increase by TBHP. a Western blot analysis of caspase 3/7 in each treatment group as described above. **b** Western blot analysis of mitochondrial and cytosolic cytochrome-c in each treatment group as described above. **c** Western blot analysis of BCL-2, and cleaved caspase-3 in each treatment group as described above. **d** Western blot analysis of Phospho-BCL-2 in each treatment group as described above. Data are expressed as the mean ± SD.

Figure 5

Knocking-down of GSK-3β protected mitochondrial apoptosis-related proteins that were induced increase by TBHP. a Western blot analysis of BCL-2, and cleaved caspase-3 in each treatment group as described above. **b** The protein levels of BCL-2, and cleaved caspase-3 in each treatment group as described above. **c**

Co-immunoprecipitation analysis of GSK-3β and BCL-2. **d** Schematic presentation illustrating the role of GSK-3β inhibitor in NPCs. Data are expressed as the mean ± SD.