Original Article

Polydatin reduces IL-1β-induced chondrocytes apoptosis and inflammatory response via p38 MAPK signaling pathway in a rat model of osteoarthritis

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Abstract: This study aimed to investigate the protective effect of polydatin on interleukin 1b (IL-1β)-induced chondrocyte apoptosis and the underlying molecular mechanisms. Chondrocytes were harvested from the joints of 4-week-old Sprague-Dawley rats, which is a characteristic feature of osteoarthritis (OA). Rat articular chondrocytes were incubated with IL-1β (10 ng/mL) in the presence of different concentrations of polydatin (20, 30 and 40 µg/mL) co-treatment for 24 h. Cell viability was evaluated by CCK-8. Apoptosis and intracellular reactive oxygen species (ROS) levels were confirmed by flow cytometry analysis. Production of TNF-α, IL-1β, IL-8 and COX-2 was evaluated by an ELISA. The quantitative real time PCR was performed to measure mRNA expression levels of Bax, Bcl-2 and MMP13. A quantitative colorimetric assay was used to determine caspase-3 activity. The contents of total p38, phosphorylated p38 (p-p38), Bcl-2, Bax and MMP13 were determined by Western blotting assay. Polydatin could inhibit IL-1β-induced chondrocyte apoptosis, ROS production, decrease of Bcl-2 and level of p-p38, and increases Bax activity, activation of Caspase-3 as well as proinflammatory cytokine productions. Additionally, p38 inhibitor (SB203580) could significantly ameliorate IL-1β-induced apoptosis in damaged chondrocytes in vitro, accompanied with reduced production of ROS and inflammation response. The present report is first to demonstrate the anti-apoptotic and anti-inflammatory activity of polydatin in human OA chondrocytes. Polydatin can effectively abrogate the IL-1β-induced injury, suggesting that polydatin may be a potential agent in the treatment of OA.

Keywords: Polydatin, intereleukin-1b, chondrocyte, p38 MAPK

Introduction

Osteoarthritis (OA) is a progressing degenerative disease that at its late stage is characterized by chondrocyte loss and degradation of the extracellular matrix [1]. Apoptosis is a highly regulated form of cell suicide and, during normal development, constitutes a physiologic mechanism by which unwanted cells are eliminated. Nevertheless, apoptosis is often involved in pathogenetic pathways. Chondrocyte apoptosis has recently been linked to OA pathogenesis by many investigators. The exact cause of OA is not known, but it is widely accepted that the inflammatory cytokines plays important roles in the development of OA, including such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [2, 3]. In particular, IL-1β has been shown to trigger apoptosis of chondrocytes by upregulation of matrix metalloproteinases (MMPs), especially collagenase (MMP-3 and -13), which degrade the extracellular matrix [4] and facilitate chondrocyte apoptosis [5]. To date, the precise interplay of the intracellular signaling pathways remains unclear, thus restricting the identification of appropriate therapeutic inhibitors for the prevention of OA.

It was reported that NO and ROS can be produced by chondrocytes in response to stimulation such as IL-1β [6, 7], which can also induce the expression of cyclooxygenase-2 (COX-2) [8]. The levels of these molecules are enhanced by IL-1β and involve upstream activation of the mitogen-activated protein kinase (MAPK) subtypes, including p38 MAPK and c-Jun N-terminal kinase (JNK). IL-1β has also been demonstrated to activate the p38 MAPK and NO pathway in
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Polydatin, a stilbene compound isolated from the dried roots of *Polygonum Cuspidatum* Sieb. et Zucc, has vast pharmacological activities, including antitumor [12], anti-platelet aggregation [13] and anti-oxidative activities [14], and possessed protective effect against sepsis [15], shock [16], ischemia/reperfusion injury [17] and congestive heart failure [18]. However, the role of polydatin in apoptosis and inflammatory responses in articular chondrocytes is still unclear. In the present study, we investigated whether polydatin exerted anti-apoptotic and anti-inflammatory activities in IL-1β-induced human OA chondrocytes.

**Materials and methods**

**Chondrocyte isolation and cultures**

Articular chondrocytes were isolated from the knee joints of 4-week-old Sprague-Dawley rats and digested with 0.25% trypsin (Gibco, Carlsbad, CA, USA) and 0.2% type II collagenase (Collagen II, Sigma Chemical Co., Poole, UK) for 30 min and 5 h at 37°C, respectively. After collection by centrifugation, chondrocytes were resuspended in DMEM supplemented with 15% FBS in culture flasks at 37°C in humidified 5% CO₂. Cells were seeded in mono-layer up to 50%-60% density and used freshly for following immunohistochemistry staining.

**Immunohistochemistry**

Previous studies pointed that expression SOX9 and Collagen II was considered as a marker for functional chondrocytes. SOX9 antibody (1:200, Abcam, ab3697) and Collagen II antibody (1:200, Abcam, ab34712) were applied accordingly for overnight and incubated with secondary antibody for 20 minutes. Those cells incubated with IgG were used as negative controls for the immunohistochemistry staining. DAB staining showed the final results of SOX9 and collagen II, and pictures were taken under the microscope at ×200 magnification. The ImageJ (NIH) image analysis system was used for quantitative analysis of positive area.

**Experimental grouping**

The primary cells were subcultured to generation 2 that were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) for at least 24 h before different treatment.

The cells were divided into five groups and treated with IL-1β in following concentrations: 0, 1, 10, 50 and 100 ng/mL. CCK-8 assay was performed 0, 24, 48 and 72 h after incubation. To investigate the protective effect of polydatin (Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China) on IL-1β (Peprotech, Rocky Hill, NJ, USA) induced cytotoxicity, the cells were divided into seven groups and were incubated with 10 ng/mL IL-1β in the absence and presence of polydatin (10, 20, 30, 40 and50 µg/mL) for 24 h. Cells in the control group were cultured without any treatment. The CCK-8 assay was performed to detect cell viability after 0, 24, 48 and 72 h.

For flow cytometry, TUNEL, ELISA, RT-PCR and Western blot experiments, the cells were divided into five groups: control, 10 ng/mL IL-1β, 10 ng/mL IL-1β+polydatin (20, 30 and 40 µg/mL) for 24 h and 10 µg/mL SB203580 (p38 MAPK inhibitor, Merck Chemicals Limited, Nottingham, UK) for 2 h prior to 10 ng/mL IL-1β for 24 h.

**Cell viability assay**

Cell viability was detected by CCK-8 assay using automicroplate reader (Infinite M200, Tecan, Austria). After incubation with various concentrations of IL-1β and/or polydatin in a 96-well plate at a density of 5×10⁵/well for indicated times, cells were incubated with 10 µL of CCK-8 for another 1 h. Absorbance at 450 nm was measured with a micro-plate reader (Bio-Rad, Hercules, CA, USA). Cells without any treatment were served as control group while triplicates were performed throughout all the procedures.

**Cell apoptosis assay**

Apoptotic cell analysis was also performed after Annexin V and propidium iodide (PI) staining of the cells by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). 5×10⁶ cells/mL were harvested and washed three times with PBS, then resuspended in binding buffer followed by
Annexin V/PI labeling at room temperature for 15 min in the dark. A total of 400 mL phosphate buffered saline (PBS) was added to the mixture and fluorescent signals quantified by flow cytometry.

**Measurement of intracellular ROS by flow cytometry analysis**

Intracellular ROS or NO was quantified by flow cytometry using DCFH-DA (Beyotime Biotechnology, Shanghai, China) staining. 5×10^5 cells/mL were stained with 10 Mm DCFH-DA for 20 min at 37°C in the dark, and re-washed with PBS three times, and subsequently 500 mL of cell suspension was added in 5 mL flow cytometry tube and analyzed quantitatively using flow cytometry. The fluorescence intensity was monitored at an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

**Measurement of LDH, SOD and NO**

At the end of the culture period, cell supernatant were collected and the production of LDH, SOD and NO was measured by Lacate dehydrogenase (LDH) assay kit (A020-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), superoxide dismutase (SOD) typed assay kit (Hydroxylamine method; A001-2, Nanjing Jiancheng Bioengineering Institute) and nitric oxide (NO) assay kit (Nitrate reductase method; A012, Nanjing Jiancheng Bioengineering Institute).

**ELISA**

At the end of the culture period, cell supernatant were collected and the concentration of TNF-α, IL-1β, IL-6 and COX-2 secreted by chondrocytes in the culture medium was further confirmed by sandwich ELISAs (R&D systems, Minneapolis, MN, USA) and all the assays were performed according to the manufacturer’s instructions.

**Quantitative real time PCR assay**

Total RNA was extracted from the cells using Trizol (Invitrogen, USA) according to the manufacturer’s protocol. The primer sequences were as follows: Bax (Forward: 5'-GGACGCATCCA-CCAAGAAG-3'; Reverse: 5'-CTGCCACACGGAAG-AAGC-3'), Bcl-2 (Forward: 5’-GGATGGCCTTT-GTGGAAC-3’, Reverse: 5’-CAACATAAGCAGT-GTAAC-3’), GAPDH (Forward: 5’-GTGGTGTCG-ACGGATTG-3’, Reverse: 5’-TCCCATTCTCAGC-CTTGAC-3’). Real-time PCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the manufacturer’s instructions. 7500 Real Time PCR System (Applied Biosystems) was employed for the thermal cycling reactions. After the normalizing with GAPDH, relative change in gene expression of GAPDH was determined by the comparative 2^ΔΔCt method.

**Western blotting**

Total protein extraction for Bax, Bcl-2, MMP13, p-AKT, AKT, GAPDH was performed according to the manufacturer’s instructions (Applygen Technologies Inc., Beijing). The concentration of protein was quantified using BCA method (Thermo Fisher Scientific Inc., USA). 15 mL proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4°C, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The signal was detected using a chemiluminescent detection system according to the manufacturer’s instructions (Pierce, Rockford, IL, USA).

**Caspase-3 activity assay**

Caspase-3 activity was measured using a Caspase-3 colorimetric assay kit (KGA203; Kaiji Biological Engineering Materials Co., Ltd, Nanjing, China) according to the manufacturer’s instructions. Briefly, cells were collected, resuspended in 50 μL of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation for 1 min (at 400×g), the supernatant was transferred to a fresh tube and then 100 μg protein was diluted in 50 μL cell lysis buffer for each assay. Samples were read at 405 nm in a plate reader Multiskan EX (Labsystems, Helsinki, Finland).

**Statistical analysis**

Results are shown as mean ± SD from at least three experiments unless stated otherwise. Statistical significance was assessed by student’s t test and one-way analysis of variance (ANOVA) using SPSS 18.0 statistical software.
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In all cases, a level of 5% was considered statistically significant (P<0.05).

Results

Effect of polydatin on IL-1β-induced chondrocyte viability

In order to study the function of polydatin in OA, we established an in vitro chondrocytes model derived from rat knee joint. Collagen II was abundantly expressed in functional chondrocytes and in joint diseases such as OA [19]. The transcription factor SOX9 has been shown to be linked to chondrocyte differentiation and induction of Collagen II synthesis [20]. The immunohistochemistry staining demonstrated a multitude of Collagen II and SOX9 was expressed in normal primary cultured chondrocytes (Figure 1A), which was indicative of a well-established chondrocytes system.

To study the effect of polydatin on IL-1β-induced chondrocyte viability, cells were first treated with increasing concentrations of IL-1β (0, 1, 10, 50 or 100 ng/mL). As shown in Figure 1B, cell viability of human OA chondrocytes was significantly decreased by IL-1β in a dose-dependent manner after 24 h (P<0.01). To check for a possible effect of polydatin on IL-1β-induced chondrocyte viability suppression, isolated primary chondrocytes were incubated with varying concentrations of polydatin (0, 10, 20, 30, 40 or 50 µg/mL) and IL-1β (10 ng/mL) for 24 h. As shown in Figure 1C, the cell viability in normal chondrocytes was significantly higher than that of cells merely exposed to IL-1β (P<0.01). The addition of polydatin (20, 30, 40 or 50 µg/mL) could reverse the reduction of the cell viability of primary chondrocytes in a dose-dependent manner (P<0.01). However, cell viability of chondrocytes was not significantly impaired by lower concentration of polydatin (10 µg/mL).

Effect of polydatin on IL-1β-induced chondrocyte apoptosis

To check for a possible effect of polydatin on IL-1β-induced chondrocyte apoptosis, isolated primary chondrocytes were incubated with...
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**Figure 2.** Effect of polydatin on cell apoptosis in IL-1β-induced rat chondrocytes. A. B. After treatment of chondrocytes with 10 ng/mL of IL-1β and different concentrations of polydatin (0, 20, 30 or 40 µg/mL) or 10 µg/mL of SB203580, the cell apoptosis was measured by flow cytometry assay. **P<0.01 vs. control, **P<0.01 vs. IL-1β treatment.
Figure 3. Effect of polydatin on ROS production in IL-1β-induced rat chondrocytes. A, B. After treatment of chondrocytes with 10 ng/mL of IL-1β and different concentrations of polydatin (0, 20, 30 or 40 µg/mL) or 10 µg/mL of SB203580, the ROS production was measured by flow cytometry assay. **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. IL-1β treatment.
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Varying concentrations of polydatin (20, 30 or 40 µg/mL) and IL-1β (10 ng/mL) for 24 h. As shown in Figure 2A and 2B, the average apoptotic rate of untreated control chondrocytes was 2.5% compared to 36.4% in chondrocytes exposed to IL-1β. The addition of polydatin showed a dose-dependent reduction of the apoptotic cells (P<0.01). The average apoptotic rates were reduced to 24.6%, 18.4% and 14.7%, respectively. Importantly, primary chondrocytes incubated with 10 µg/mL SB203580 (p38 MAPK inhibitor) 2 h prior to the addition of IL-1β (10 ng/mL) for 24 h also reduced the apoptotic rate to 11.3% compared with IL-1β treatment (Figure 2, P<0.01).

**Effect of polydatin on IL-1β-induced ROS production in chondrocytes**

The effect of the polydatin on IL-1β-induced ROS production was shown in Figure 3A and 3B. Chondrocytes were treated with IL-1β (10 ng/mL) and polydatin (20, 30 or 40 µg/mL) for 24 h. Stimulation with IL-1β significantly increased the production of ROS by 10.5-fold compared to un-stimulated control (P<0.01). Importantly, there was a significant downregulation in ROS production in IL-1β-stimulated chondrocyte cultures that were treated with the polydatin (20, 30 or 40 µg/mL). The ROS production was decreased by 16.6%, 39.9% and 61.8%, respectively (Figure 3A and 3B, P<0.05, P<0.01). In parallel, 10 µg/mL SB203580 significantly decreased the ROS production by 71.9% induced by IL-1β (P<0.01).

**Effect of polydatin on IL-1β-induced production of LDH, SOD and NO in chondrocytes**

The concentration of LDH, SOD and NO in IL-1β-induced chondrocytes was shown in Figure 4A-C. Stimulation with IL-1β significantly increased the concentration of LDH and NO by 1.3-fold and 2.3-fold, respectively, and decreased the concentration of SOD by 67.3%, compared to un-stimulated control (P<0.01). The addition of polydatin (20, 30 or 40 µg/mL) could reverse the changes of the production of LDH, SOD and NO in primary chondrocytes in a dose-dependent manner (P<0.05, P<0.01).
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Importantly, 10 µg/mL SB203580 mimics the effect of polydatin on IL-1β-induced production of LDH, SOD and NO in chondrocytes (P<0.01).

**Effect of polydatin on IL-1β-induced proinflammatory cytokine in chondrocytes**

The concentration of proinflammatory cytokines, including TNF-α, IL-1β, IL-8 and COX-2 was quantitatively determined by ELISA. We found that stimulation with IL-1β significantly increased the concentration of TNF-α, IL-1β, IL-8 and COX-2 by 2.1-fold, 1.8-fold, 1.9-fold and 2.1-fold, respectively, compared to unstimulated control (Figure 5A-C, P<0.01). Polydatin exhibited a broad spectrum of inhibitory effect on the concentration of proinflammatory cytokines. Polydatin significantly reduced the concentration of TNF-α, IL-1β, IL-8 and COX-2 induced by IL-1β in a dose-dependent manner (Figure 5A-C, P<0.01). Importantly, 10 µg/mL SB203580 mimics the effect of polydatin on IL-1β-induced production of TNF-α, IL-1β, IL-8 and COX-2 in chondrocytes (Figure 5A-C, P<0.01).

**Effect of polydatin on IL-1β-induced protein in chondrocytes**

To further investigate the molecular mechanism through which polydatin protects chondrocytes from IL-1β-induced apoptosis and inflammatory response, Real-time PCR and Western blot analyses were performed to study changes in Bax, Bcl-2, MMP13 and p38 MAPK signaling. As shown in Figure 6A-C, the mRNA expression of Bax and MMP13 was significantly increased by IL-1β stimulation, and Bcl-2 mRNA expression was decreased (P<0.01). Polydatin significantly reversed the mRNA expression of Bax and MMP13 induced by IL-1β in a dose-dependent manner (Figure 6A-C, P<0.01). 10 µg/mL SB203580 mimics the effect of polydatin on IL-1β-induced changes in Bax, Bcl-2 and MMP13 mRNA levels in chondrocytes (Figure 6A-C, P<0.01). Also, the Real-time PCR...
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results were similar to that measured by Western blotting analysis (Figure 6D, P<0.01). As shown in Figure 6D, p-p38 levels were significantly increased after 24 h with IL-1β stimulation compared to the control group (P<0.01). Notably, treatment with polydatin or SB203580 significantly decreased p-p38 expression in IL-1β-stimulated chondrocytes (P<0.01). Total p38 levels were unchanged. Additionally, as shown in Figure 6E, addition of polydatin or SB203580 significantly decreased p-p38 expression in IL-1β-stimulated chondrocytes (P<0.01). These results suggest that polydatin exerts its effects via decreased p38 MAPK signaling.

Discussion

Polydatin has shown therapeutic effect on ischemia/reperfusion-induced apoptosis [17], fulminant hepatic failure [21] and platelet aggregation [22]. However, the effects of polydatin in OA have not yet been reported. Our present findings indicate that treatment with polydatin in IL-1β-induced apoptosis of chondrocyte promotes cell proliferation and Bcl-2 expression, inhibits ROS production and expression of Bax and MMP13, and reduces Caspase-3 activation. These effects by polydatin are partially mediated via suppressed p38 MAPK signaling.

Proinflammatory cytokines such as IL-1β can induce chondrocyte apoptosis, which is closely related to the occurrence and development of OA [23]. In vitro studies have confirmed that Caspase-3 activity is significantly increased in IL-1β-induced chondrocyte apoptosis. Similar results were also demonstrated by Huang et al [24]. They found that IL-1β-induced chondrocyte apoptosis through decreased the expression levels of Bcl-2/Bax ratio and increased Cyt c expression and Caspase-3 activation. The present study provides first hand evidence that polydatin (20, 30 and 40 µg/mL) treatment on IL-1β-induced apoptosis increased the Bcl-2/Bax ratio and inhibited Caspase-3 activity.

Excess generation of ROS can activate diverse signaling pathways and result in oxidative stress and inflammatory response. Endogenous antioxidant enzymes, such as glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD), can eliminate the oxygen-derived free radicals and reduce the oxidative stress caused by ROS [25]. NO can induce intracellular signal transduction and inflammatory gene activation. In chondrocytes, NO can inhibit synthesis of collagens and proteoglycans and increase MMP activity [1]. In our study, polydatin could inhibit the release of ROS, SOD and NO induced by IL-1β in chondrocyte.

In addition to inhibiting chondrocyte apoptosis, drugs used to treat OA are anticipated for promoting chondrocyte matrix synthesis. Previous study has shown that IL-1β-induced upregulation of MMP13 is a key event in the irreversible breakdown of cartilage matrix via digestion of Collagen II and the consequent release of matrix proteoglycans from the cartilage leads to destructive joint diseases [26]. Interestingly, in the present study, we demonstrated that polydatin prevented IL-1β-induced MMP13 expressions at the mRNA and protein levels in human OA chondrocytes. Previous studies showed that IL-1β is an important regulator in the induction of proinflammatory cytokines in chondrocytes [1, 27]. In the current study, polydatin inhibited IL-1β induced TNF-α, IL-6 and COX-2 expression in a concentration-dependent manner.

We also investigated the molecular mechanisms by which polydatin inhibited apoptosis and inflammatory mediators in response to IL-1β in chondrocytes. We focused on the effects of polydatin on p38 MAPK due to its importance in apoptosis and inflammatory process [28, 29]. Indeed, a previous study utilizing SB203580, a selective inhibitor of p38 MAPK, confirms the present findings which showed increased cell proliferation and inhibition of NO release in chondrocytes which had been cultured with IL-1β [10].

In summary, as a pro-inflammatory cytokine, IL-1β showed its remarkable strength in deteriorating chondrocytes functions, in particularly apoptosis, in an in vitro OA model. Polydatin was inevitably corrected IL-1β-induced apoptotic impact and inflammation in chondrocyte partially through p38 MAPK signaling pathway, and thus might be considered as a promising therapeutic target during OA therapy.

Disclosure of conflict of interest

None.

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References


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