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Original Article

Ginsenoside Rg3 exhibits anti-catabolic and anti-apoptotic effects in IL-1β treated human disc nucleus pulposus cells and in a rat model of disc degeneration by inactivating the MAPK pathway

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Abstract

Intervertebral disc degeneration (IDD) is the major cause of degeneration of joint diseases. IDD is characterized by a large number of apoptosis of nucleus pulposus cells (NPCs) and extracellular matrix (ECM) degradation. Ginsenoside Rg3 is the active component extracted from ginseng and has a vital function in modulating diseases. This study aimed to investigate the regulatory functions of ginsenoside Rg3 in IDD. We established the IDD cell model via inducing NPCs with IL-1β. The rat model of IDD was established by fibrous ring puncture method. Cell apoptotic capability was assessed through TUNEL assay. The levels of catabolic proteases MMPs and ADAMTSs were tested by western blot and RT-qPCR. IL-1β induction notably promoted the apoptosis of NPCs, while ginsenoside Rg3 treatment reversed the promoting function of IL-1β. Furthermore, we found that MMP2, MMP3, Adamts4, and Adamts5 levels were increased in IL-1β-induced NPCs, while ginsenoside Rg3 treatment markedly reduced their levels. Additionally, ginsenoside Rg3 was found to suppress the IL-1β-stimulated p38 MAPK pathway in NPCs. In the IDD rat model, we found that ginsenoside Rg3 treatment can alleviate NPC degeneration, recover the arrangement of annulus fibrous, and preserve more proteoglycan matrix. Moreover, ginsenoside Rg3 reduced apoptosis and catabolism and inactivated the p38 MAPK pathway in IDD rats. Ginsenoside Rg3 exhibits anti-catabolic and anti-apoptotic effects in IL-1β-stimulated NPCs and IDD rats by inactivating MAPK pathway.

Keywords: Intervertebral disc degeneration; Ginsenoside Rg3; Nucleus pulposus cells; MAPK pathway

1. Introduction

Intervertebral disc degeneration (IDD) is the main cause of degeneration of joint diseases including cervical spondylosis and lumbar disc herniation (1, 2). The intervertebral disc is composed of upper endplate, center nucleus pulposus and outer annulus fibrosus (3). With age, the intervertebral disc gradually loses its flexibility, elasticity and shock absorption function, and the fibrous ring around the intervertebral disc becomes fragile (4). The main cells in the nucleus pulposus tissues are nucleus pulposus cells (NPCs), which play important roles in extracellular matrix (ECM) degradation (3). The degeneration mainly displays as a large number of the NPC apoptosis and ECM degradation (3, 5). IDD is caused by assorted risk factors, such as family heredity, mechanical trauma, and ageing (6). The inflammation process is commonly regarded as key event in IDD (7). Interleukin (IL)-1β is the most important inflammatory cytokine and has been recognized as a major risk factor for IDD (4). On the one hand, IL-1β can cause damage to NPCs by triggering the production of various pro-inflammatory mediators (8). On the other hand, it can increase matrix degradation and reduce matrix synthesis, thereby exacerbating the progression of IDD (1). Evidence has confirmed that high levels of IL-1β can induce apoptosis of NPCs in IDD [5, 6]. Nevertheless, existing treatments cannot fundamentally cure IDD. Thus, finding new agents to cure IDD is particularly important.

Ginsenoside is a vital active pharmaceutical component isolated from the traditional Chinese medicine ginseng, and it has been utilized for therapeutic purposes due to as anti-inflammatory, anti-stress, anti-oxidation, and anti-tumor effects (9). Rg3 is considered one of the most biologically active extracts among ginsenosides (10). More and more studies have revealed Rg3 exerts its therapeutic roles in human diseases. For example, ginsenoside Rg3 is reported to ameliorate acute pancreatitis through the activation of the NRF2/HO-1-mediated ferroptosis pathway (11). Ginsenoside Rg3 can attenuate ovariectomy-induced osteoporosis through AMPK/mTOR signaling pathway (12). Ginsenoside Rg3 can alleviate aluminum chloride-induced bone damage (13). Ginsenoside Rg3 can protect chondrocytes via inhibiting chondrocyte senescence in the IL-1β-induced chondrocytes (14, 15). Furthermore, it has been confirmed that ginsenoside Rg3 attenuates TNF-α-

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induced injury of NPCs through the inactivation of NF-κB signal (16). Therefore, the effect of ginsenoside Rg3 on IL-1β-induced NPCs is worth exploring in depth.

In this study, NPCs induced by IL-1β was established as the IDD cell model. The impacts of ginsenoside Rg3 treatment on IL-1β-induced injury of NPCs were explored, and our discoveries may offer a new treatment to alleviate IDD process.

2. Materials and methods

2.1. Cell culture

The human NPCs (CP-H097; Procell; Wuhan, China) were incubated in DMEM/F12 medium with 10% FBS. IL-1β (20 ng/mL; Sigma-Aldrich, USA) was utilized for treating NPCs for 24 h to induce the IDD cell model (17). Ginsenoside Rg3 (50 μg/mL; Thermo Fisher Scientific, USA) was utilized for treating NPCs. After 24 h, cells were collected for assay.

2.2. CCK-8 assay

Cell viability was determined utilizing CCK-8 kit (Sigma-Aldrich). Cells were put in 96-well plate for 24 h of incubation. After treatment of IL-1β and ginsenoside Rg3, 10% CCK-8 reagents were implemented to each well for the additional 2 h. The absorbance at 450 nm was assessed through the microplate reader (Boehringer Mannheim ES700, UK).

2.3. TUNEL assay

The treated cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 5 min and rinsed with PBS. Next, they were dyed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, Beyotime, China). Nucleus were stained by DAPI. The photograph was observed by the fluorescence microscope (Olympus).

2.4. Western blot

NPCs and tissues were lysed in the RIPA buffer. Then, proteins were isolated by 12% SDS-PAGE and transferred onto PVDF membranes. Later, the membranes were rinsed and blocked by 5% skim milk powder for 2 h, and then cultured with the appropriate primary antibodies (Abcam, USA) at 4 °C for one night. Afterwards, membranes were further cultured with secondary antibodies (Abcam) for 2 h. Then, ECL luminescent liquid was utilized for visualizing the proteins and images were captured through the gel imaging system. The protein grey density was quantified through the ImageJ software.

2.5. RT-qPCR

Total RNA was subjected to extraction from cells or tissues utilizing the TRizol method. Then, RNA concentration was tested by NanoDrop, and cDNA was reversely transcribed by the primer reverse transcription kit (Thermo Fisher). qPCR was carried out by SYBR Premix ExTaqTM II (Takara) on StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Gene expression was determined by $2^{-\Delta\Delta Ct}$ method normalized to GAPDH.

2.6. Immunofluorescence assay

NPCs were incubated in 24-well plates for treatment and then fixed with 4% paraformaldehyde. Then cells were rinsed with PBS and cultured with 0.2% Triton X-100 for permeabilization. After blocked with 5% goat serum, cells were cultured with the primary antibody against p38 (Abcam) for one night at 4°C. After that, cells were cultured with FITC-conjugated secondary antibodies for an additional 1 h. Finally, the fluorescence microscope (Olympus) and Image-Pro Plus 6.0 software were utilized for observation and quantification.

2.7. The establishment of rat IDD model

The animal experiment was approved by the Ethics Committee of Siyang County Hospital of Traditional Chinese Medicine. Adult SD rats (n=30; male; 200–300 g) were purchased from the Siyang County Hospital of Traditional Chinese Medicine and fed for seven days to adapt to the environment. The IDD model was established utilizing the fibrous ring puncture method (18). Rats were randomly divided into three groups (n = 10): the control group, the IDD group, and IDD+Rg3 group. Rats were subjected to anesthesia with 2% pentobarbital. After sterilization with povidone-iodine, the coccygeal intervertebral disc (Co8-9) was percutaneously punctured through the 21 G needle at a depth of 5 mm, followed by rotating at 360° and keeping for 30 s. Rats in IDD+Rg3 group were intraperitoneally injected Rg3 (20 mg/kg/d) for 4 weeks. The control rats were received with an equivalent volume of physiological saline. After that, rats were euthanized with excessive pentobarbital.

2.8. X-ray

The X-ray film analyzed through the X-ray system (United Imaging, China) was employed for evaluating the intervertebral space height of rats. Then the Disc Height Index (DHI) was calculated.

2.9. Histological analysis

Rats were euthanized and tails were gathered for experiments. Tissues were fixed by 4% formaldehyde, decalcified with 10% EDTA solution, and embedded in paraffin, followed by cutting into 5-μm sections. Then sections were dyed with Hematoxylin and Eosin (H&E) or Safranin-O/Fast Green. The improved histopathological grading score system was applied to evaluate the degree of IDD of rats.

2.10. Statistical analyses

Statistical data analyzed by GraphPad Prism 6.0 were presented as means ± SD from three individual repeats. Data were analyzed by Student's *t*-test or one-way ANOVA to compare two or multiple groups. $P<0.05$ represented statistical significance.

3. Results

3.1. Ginsenoside Rg3 exhibits anti-catabolic and anti-apoptotic effects in IL-1β-treated human NPCs

Ginsenoside Rg3 has been confirmed to exert the regulatory function in human diseases (11-16), thus we detected its function in IDD. The 2D chemical structural formula of ginsenoside Rg3 was obtained from pubchem website and shown in Figure 1A. Then, we detected the impacts of ginsenoside Rg3 on human NPCs. NPCs were induced by 20 ng/mL IL-1β to establish the NPC degeneration model. Ginsenoside Rg3 (50 μg/mL) was utilized to treat IL-1β-stimulated NPCs for detecting its functions. Through CCK-8 assay, we discovered that, in comparison to control cells, the viability of IL-1β-stimulated

NPCs was significantly reduced, while ginsenoside Rg3 treatment recovered the reduced viability (Figure 1B). It was manifested by the TUNEL assay that TUNEL-positive NPCs were increased by IL-1β induction. However, ginsenoside Rg3 treatment reversed this promoting effect, suggesting the anti-apoptotic effect of ginsenoside Rg3 on IL-1β-induced NPCs (Figure 1C). Furthermore, apoptosis-related proteins were tested through western blot and RT-qPCR. The outcomes manifested that Bax levels promoted in the IL-1β group were reduced in the IL-1β+Rg3 group, whereas Bcl-2 levels declined in IL-1β group were elevated in the IL-1β+Rg3 group (Figure 1D-E). MMPs and ADAMTSs have been confirmed as the two main families of catabolic proteases (19), thus we further evaluated the level changes of their main proteins in NPCs of different groups. We observed that both protein and mRNA levels of MMP2, MMP3, Adamts4, and Adamts5 were elevated in IL-1β-induced NPCs, while ginsenoside Rg3 treatment markedly reduced their levels (Figure 1F-G). Overall, ginsenoside Rg3 exhibited anti-catabolic and anti-apoptotic effects in IL-1β-induced NPCs.

3.2. Ginsenoside Rg3 suppresses the IL-1β-stimulated p38 MAPK pathway in human NPCs

MAPK pathway has been confirmed to be related to IDD pathogenesis and its abnormal activation can deteriorate IDD process (20). For the sake of further exploring the regulatory mechanism of ginsenoside Rg3 in IL-1β-induced NPCs, we tested the levels of main protein p38 in MAPK signaling. Through western blot, IL-1β stimulation markedly elevated the phosphorylated p38 levels in NPCs, while its levels were reduced by ginsenoside Rg3 in IL-1β-induced NPCs (Figure 2A). Furthermore, it was manifested by IF staining that the fluorescence intensity of p38 was enhanced in the IL-1β-stimulated NPCs but ginsenoside Rg3 treatment abolished this promoting effect and reduced p38 fluorescence (Figure 2B). Overall, ginsenoside Rg3 suppressed MAPK pathway in IL-1β-stimulated NPCs.

3.3. Ginsenoside Rg3 inhibits the degree of IDD in rats

The effects of ginsenoside Rg3 in IDD were further explored through the IDD rat model. X-ray outcomes exhibited that the DHI of the caudal vertebrae of IDD rats was lower than that of control rats, while the DHI of ginsenoside Rg3-tretaed IDD rats was markedly elevated in comparison of the IDD model rats (Figure 3A). It suggested that ginsenoside Rg3 can improve the intervertebral disc height of the IDD rats. Through HE staining, we observed a decrease in quantity of NPCs and a disorderly arrangement of annulus fibrous in intervertebral disc tissues of the IDD rats. Nevertheless, in comparison to IDD model group, ginsenoside Rg3 treatment notably relieved NPC degeneration and recovered the arrangement of annulus fibrous (Figure 3B). The histological score showed that the score value was increased in IDD group but reduced in IDD+Rg3 group (Figure 3B). Moreover, the Safranin-O/Fast Green staining demonstrated that proteoglycan matrix was markedly declined in IDD group in comparison of control group, while proteoglycan matrix was well preserved in IDD+ginsenoside Rg3 group compared with IDD group (Figure 3C). Thus, we confirmed that ginsenoside Rg3 inhibited the degree of IDD in rats.

3.4. Ginsenoside Rg3 reduces apoptosis and catabolism in IDD rats

The function of ginsenoside Rg3 on apoptosis and catabolism in IDD rats was further investigated. Western blot and RT-qPCR outcomes revealed that Bax levels were increased in intervertebral disc tissues of the IDD rats while decreasing by ginsenoside Rg3 administration. However, Bcl-2 levels showed the opposite trend to Bax levels in tissues (Figure 4A-B). We further observed that MMP2, MMP3, Adamts4, and Adamts5 levels elevated in the IDD group relative to control group, while their levels reduced in the IDD+ginsenoside Rg3 group compared with IDD group (Figure 4C-D). Overall, ginsenoside Rg3 reduced apoptosis and catabolism in IDD rats.

3.5. Ginsenoside Rg3 inactivates the p38 MAPK pathway in IDD rats

The activity of p38 MAPK pathway in IDD rats was

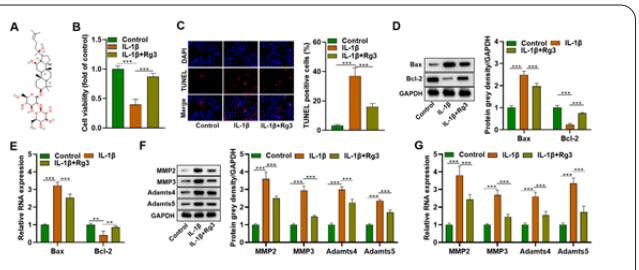

Fig. 1. Ginsenoside Rg3 exhibits anti-catabolic and anti-apoptotic effects in IL-1β-treated human NPCs. (A) The 2D chemical structural formula of ginsenoside Rg3 was obtained from pubchem website. (B) The viability of human NPCs in the control group, the IL-1β group, and the IL-1β+ginsenoside Rg3 group was tested by CCK-8 assay. (C) TUNEL assay was implemented for testing cell apoptosis of NPCs with different treatments. (D-G) Western blot and RT-qPCR outcomes of Bax, Bcl-2, MMP2, MMP3, Adamts4, and Adamts5 levels in NPCs with different stimuli. $^{**}p<0.01$, $^{***}p<0.001$.

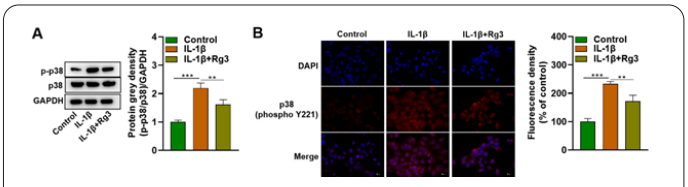

Fig. 2. Ginsenoside Rg3 suppresses the IL-1β-stimulated p38 MAPK pathway in human NPCs. (A) Western blot outcomes of p-p38 and total p38 levels in human NPCs of the control group, the IL-1β group, and the IL-1β+ginsenoside Rg3 group. (B) IF assay was utilized for testing p38 levels in NPCs. $^{**}p<0.01$, $^{***}p<0.001$.

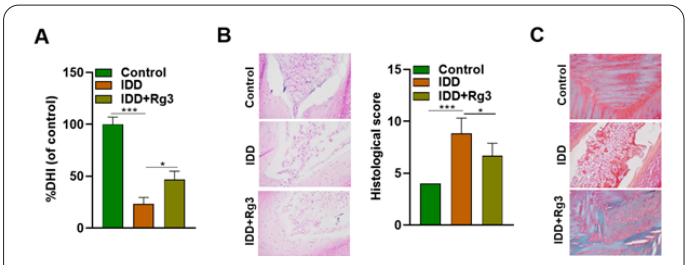

Fig. 3. Ginsenoside Rg3 inhibits the degree of IDD in rats. (A) The semiquantitative analysis of X-ray images of the Co8-9 discs in different rats. (B) HE staining assay was utilized for determining the histopathology of in intervertebral disc tissues of the control group, IDD group, and IDD+ginsenoside Rg3 group. (C) Safranin-O/Fast Green staining was performed for measuring proteoglycan deposition in intervertebral disc tissues. $^{*}p<0.05$, $^{***}p<0.001$.

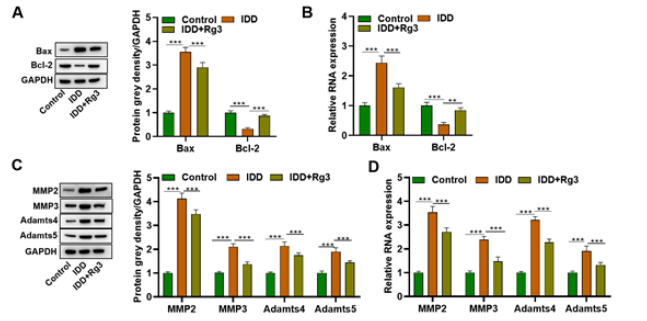

Fig. 4. Ginsenoside Rg3 reduces apoptosis and catabolism in IDD rats. (A-B) Western blot and RT-qPCR outcomes of Bax and Bcl-2 levels in intervertebral disc tissues of the control group, IDD group, and IDD+ginsenoside Rg3 group. (C-D) Western blot and RT-qPCR outcomes of MMP2, MMP3, Adamts4, and Adamts5 levels. $^{**}p<0.01$, $^{***}p<0.001$.

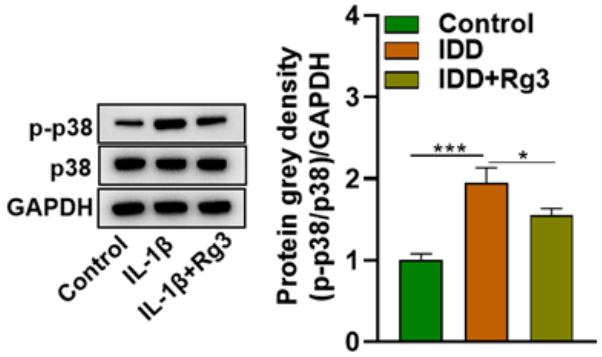

Fig. 5. Ginsenoside Rg3 inactivates the p38 MAPK pathway in IDD rats. (A) Western blot outcomes of p-p38 and total p38 levels in intervertebral disc tissues of the control group, IDD group, and IDD+ginsenoside Rg3 group. $^{*}p<0.05$, $^{***}p<0.001$.

further determined. Western blot outcomes manifested that the phosphorylated p38 levels were increased in intervertebral disc tissues of the IDD rats, while ginsenoside Rg3 administration notably reduced phosphorylated p38 levels (Figure 5A). Thus, ginsenoside Rg3 inactivated the p38 MAPK pathway in IDD rats.

4. Discussion

IDD is a common orthopedic disease. At present, common treatment methods include conservative treatment and surgical treatment, but the treatment effect is not ideal (1, 2). Thus, investigating novel agents for treating IDD has become a vital research direction. NPC dysfunction can disturb the equilibrium between ECM synthesis and decomposition, which is crucial for maintaining the physiological function of intervertebral discs (21). IL-1β is an important inflammatory cytokine and is highly expressed in degenerative intervertebral disc tissues (4). It can take part in assorted pathological progresses, such as inflammatory reaction, matrix destruction, and cellular apoptosis (4). IL-1β-induced NPCs have been extensively utilized for simulating IDD cell injury. Ginsenoside Rg3 is the main active ingredient of ginseng, and it has been confirmed to exert assorted protective functions, including antioxidant, anti-inflammatory, and anti-aging effects in human diseases (22-25). In this study, we constructed an injury model of NPCs using IL-1β and tested the therapeutic effect of ginsenoside Rg3 on the injured NPCs. Excessive apoptosis of NPCs is one of the main characteristics of IDD (26). Our study found a notable promotion in the apoptosis rate of NPCs stimulated by IL-1β. The expression level of pro-apoptosis protein Bax notably elevated under the induction of IL-1β, while the level of anti-apoptosis protein Bcl-2 markedly decreased, indicating that IL-1β induction accelerated the apoptosis of NPCs. However, ginsenoside Rg3 treatment reduced the apoptosis rate, declined Bax level, and elevated Bcl-2 level in IL-1β-stimulated NPCs, indicating that ginsenoside Rg3 played an anti-apoptotic role in IDD. Furthermore, we constructed an IDD rat model and observed upregulation of pro-apoptotic protein levels in intervertebral disc tissues, but this upregulation was inhibited by Rg3 administration. Previous studies have confirmed that ginsenoside Rg3 can reduce TNF-α-induced injury in chondrocytes through the anti-apoptotic and anti-inflammatory mechanisms (27). Ginsenoside Rg3 alleviates kidney injury through inhibition of apoptosis and autophagy-inhibited NLRP3 (28). Ginsenoside Rg3 promotes cardiac function after myocardial ischemia/reperfusion through suppressing cell apoptosis (29). These studies further supported our findings.

IDD is usually accompanied by ECM degradation and it is caused by the disequilibrium of ECM synthesis and catabolism (30). Catabolism is primarily modulated through proteolytic enzymes, of which matrix metalloproteinases (MMPs) and disintegrin and metalloproteinases with thrombospondin motif proteins (ADAMTSs) exert the crucial function (19). A flow of evidence demonstrated many members of MMPs and ADAMTSs express at the high levels in degenerative intervertebral disc tissues and are closely involved in ECM decomposition and IDD progression (31, 32). Additionally, evidence shows that IL-1β can enhance ECM degradation during IDD by inducing the production of MMPs and ADAMTSs (33). It is reported that Adamts4 and Adamts5 are notably increased in loaded NPCs (34). In this study, we found that MMP2, MMP3, Adamts4, and Adamts5 expression levels were elevated in the IL-1β-stimulated NPCs and intervertebral disc tissues of IDD rats, while ginsenoside Rg3 treatment markedly reduced their levels. Previously, it was reported that Rg3 suppressed bone injury via alleviating structural damage to the femur, inhibiting cell apoptosis, and promoting ECM synthesis (13). In summary, ginsenoside Rg3 exhibited anti-catabolic and anti-apoptotic effects in IL-1β-stimulated NPCs and IDD rats.

MAPK pathway exerts a vital function in signal transduction cascade modulating inflammatory reaction (35). The p38 MAPK pathway belongs to MAPK superfamily, mediating the modulation of assorted physiological and pathological processes including inflammation, development, and apoptosis (36). In recent years, a flow of research efforts has revealed the importance of p38 MAPK pathway in IDD (37, 38). It is reported that activation of the p38 MAPK pathway aggravates IDD process via facilitating NPC apoptosis and stimulating inflammatory response (39, 40). Quercetin has been confirmed to alleviate IDD development by inhibiting p38 MAPK pathway (41). In this study, we proved that the phosphorylated p38 levels were elevated in NPCs and the intervertebral disc tissues of the IDD rats, while ginsenoside Rg3 administration notably reduced phosphorylated p38 levels. Thus, ginsenoside Rg3 was proved to inactivate the p38 MAPK pathway in IDD.

Taken together, this study confirms that ginsenoside Rg3 exhibits anti-catabolic and anti-apoptotic effects in IL-1β-treated human NPCs and in a rat model of IDD by inactivating the p38 MAPK pathway. This finding suggests that ginsenoside Rg3 may become a promising agent for treating IDD.

Informed Consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
CJ and ZB conducted the experiments and wrote the paper; WL and XJ analyzed and organized the data; CJ conceived, designed the study and revised the manuscript.

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