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Sirt1 suppresses MCP-1 production during the intervertebral disc degeneration by inactivating AP-1 subunits c-Fos/c-Jun

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Abstract. – OBJECTIVE: The anti-inflammatory effect of Sirtuin 1 (Sirt1) during intervertebral disc degeneration (IDD) has been widely confirmed. Monocyte chemoattractant protein-1 (MCP-1) activation is the initiating inflammatory response associated with the IDD. However, whether Sirt1 suppresses MCP-1 in the intervertebral disc is unclear.

PATIENTS AND METHODS: The MCP-1 and Sirt1 protein expression in the degenerated and non-degenerated NP tissues were compared by immunohistochemistry (IHC). We induced nucleus pulposus (NP) cell degeneration by IL-1β and mediated cellular Sirt1 expression through the Sirt1 activator resveratrol (Res) or inhibitor Nicotinamide (Nico). In addition, the inhibitors of MCP-1 and Activator protein 1 (AP-1) were also used in cell culture. The function of NP cells was determined by the type II collagen and Cell Counting Kit-8 (CCK-8) assay. We assessed the Sirt1 and MCP-1 expression by the Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR). The AP-1 activity was valued by the phosphorylation of its components c-Fos, and c-Jun.

RESULTS: Both *in vivo* and *in vitro* experimental results indicated that MCP-1 was upregulated in the degenerated condition, which was opposite to Sirt1 expression. Res suppressed AP-1, the phosphorylation of c-Fos/c-Jun, and the MCP-1 expression. On the contrary, Sirt1 downregulation by Nico aggravated the phosphorylation of c-Fos/c-Jun and MCP-1 expression. However, the MCP-1 suppression did not affect the Sirt1 and AP-1 levels. The destruction of AP-1 activation also inhibited MCP-1 expression but not Sirt1. The upregulation of Sirt1 and suppression of MCP-1 improved the type II collagen expression and cell viability, which was injured by IL-1β.

CONCLUSIONS: Sirt1 suppresses the MCP-1 production in the degenerated NP cells by suppressing the phosphorylation of the AP-1 subunits c-Fos and c-Jun.

Key Words:
Intervertebral disc degeneration, Sirt1, MCP-1, AP-1, c-Fos, c-Jun.

Introduction

Low back pain (LBP) brings great pain to patients, and most people experience LBP throughout their lives. Intervertebral disc degeneration (IDD) is one of the main pathological factors of low back pain[1]. With the aggravation of IDD, the expression of inflammatory factors in intervertebral disc tissue gradually increased. The activation of inflammatory factors promotes the catabolism and changes the structure of intervertebral disc and further exacerbates the its degeneration[2]. On the other hand, inflammatory factors stimulate intervertebral disc cells to produce pain-related substances, such as nerve growth factor (NGF), substance P, and prostaglandins, which aggravate patients' pain[3]. It is clear that the appearance of inflammatory factors and inflammatory reactions playing a vital role in the pathogenesis of low back pain was caused by disc degeneration[4].

Chemokines are a class of small-molecule cytokines that can chemotactically recruit the cells to direct movement. Chemotaxis is widely involved in pathological processes containing cell growth, inflammation, tumor occurrence, and wound re-

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pair[5,6]. Chemokines are divided into four categories due to the different arrangements of cysteine, namely C, CC, CXC, CX3C. Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the CC subfamily, which can recruit the appearance and aggregation of inflammatory factors, such as macrophages, monocytes, and interleukin 1 (IL-1)[7,8]. MCP-1 is likely to be the most critical initiating factor that triggers the inflammatory response in the intervertebral disc tissue[9].

Silent mating type information regulation 2 homologs (sirtuins), which depend on NAD+ deacetylase, have various biological functions, such as regulating cell senescence, proliferation, differentiation, apoptosis, and metabolism. There are 7 members in the sirtuins family, containing sirtuins 1-7[10]. Sirt1 stands for sirtuin 1, not only involves cell growth and apoptosis but also functions as an inflammation inhibitor. In periodontal ligament, Sirt1 overexpression reduces the IL-1β, IL-6, and TNF-α production through the suppression of TLR4 and JNK/NF-κB pathway[11]. It prevents acute gouty inflammation *via* regulating macrophage polarization through the PI3K/Akt/STAT6 pathway[12]. Besides, Sirt1 also suppresses IL-1β induced inflammatory during nucleus pulposus (NP) cells degeneration by the TLR2/SIRT1/NF-κB pathway[13]. Sirt1 has played an efficiently anti-inflammatory role in various organizations. However, the anti-inflammatory effect of Sirt1 in IDD is still poorly understood, especially the effect on MCP-1.

Activator protein 1 (AP-1) is a nuclear transcription factor that regulates cell apoptosis, growth, and inflammation. Its structure is a heterodimer composed of proteins, mainly belonging to the c-Fos and c-Jun families[14,15]. The phenotype of articular chondrocytes (CHs) is similar to that of NP cells. In CHs, AP-1 regulates the cellular inflammation and controls the progress of arthritis[16]. Apart from this, Sirt1 is reported to be involved in regulating AP-1 activity to suppress matrix metalloproteinase 13 (MMP13) expression[17] or protecting aortic dissection[18]. MCP-1 is the target gene of AP-1 in various cells. The activity of MCP-1 in the NP may be related to the activation of AP-1[19]. Therefore, we speculated that Sirt1 might inhibit the expression of MCP-1 by regulating AP-1 and exert the anti-inflammatory effect on the intervertebral disc. In this study, we used the primary NP cells extracted from the NP tissue of patients with lumbar disc herniation to clarify the mechanism of Sirt1 inhibiting MCP-1. What we found provides a novel insight into the anti-inflammatory role of Sirt1 in IDD.

Patients and Methods

Source of NP Tissue

The NP tissue of the lumbar intervertebral disc was taken from the intraoperative NP specimens of patients with lumbar spinal trauma (as control) or herniated intervertebral discs (as IDD) who were hospitalized in the Orthopedics Department of our hospital from November to December 2019. We recruited five patients from each group. The inclusion criteria of the control group were patients without long-term lumbar and leg pain before the injury, and no significant IDD confirmed by CT and MRI. The inclusion criteria of the IDD group were that patients with typical clinical symptoms and signs, and lumbar disc herniation confirmed by CT and MRI examinations. The specimens were divided into 2 parts; one part was used for NP cell extraction, while the other part was prepared for paraffin section for immunohistochemistry (IHC). Before the specimens were taken from both groups, the patients were informed and signed an informed consent form. This project was reviewed and approved by the Ethics Committee of Hainan Affiliated Hospital of Hainan Medical University.

NP Cells Extraction

The specimens removed from the patients were reserved in the culture medium and brought to the lab immediately. We extracted the NP cells from the NP tissue specimens without IDD as the following protocol. NP tissues were cut into fragments and washed with sterile phosphate-buffered saline (PBS). The fragments were digested with type II collagenase (0.25%, Sigma-Aldrich, St. Louis, MO, USA) overnight. The cell pellets were collected after centrifuge and resuspended in the culture medium [Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, Gibco, Rockville, MD, USA]. NP cells were seeded in a six-well plate with a density of 1×10^4 /well, and the medium was changed every three days.

Drug Treatments

To induce the NP cells degeneration *in vitro*, we treated them with a ranged concentration of IL-1β (5, 10, and 20 ng/mL). Resveratrol[20] (Res, Sigma-Aldrich, St. Louis, MO, USA) and Nicotinamide[21] (Nico, Sigma-Aldrich, St. Louis, MO, USA) were used to up-regulate or down-regulate the Sirt1 expression of NP cells. Besides, the

MCP-1 inhibitor Bindarit[22] (Bin, Selleck, Houston, TX, USA) and SR11302[23] the inhibitor of AP-1 (SR, Santa Cruz, Santa Cruz, CA, USA) were also added in the NP cells culture.

Immunohistochemistry (IHC)

Half part of NP samples was used to analyze the MCP-1 and Sirt1 expression of both groups. The samples were fixed with 4% formalin for 24 h, and then, dehydrated with gradient ethanol, cleared with xylene, and embedded in paraffin. The Paraffin specimens were sectioned in 5 μm-thick and dewaxed with xylene and hydrated with gradient alcohol for IHC. After blocked with 5% goat serum solution for 10 min, the sections were incubated with the primary antibody against MCP-1 (ab9669, Abcam, Cambridge, MA, USA) and Sirt1 (ab110304, Abcam, Cambridge, MA, USA) at 4°C overnight. The following day, the biotinylated goat anti-rabbit IgG and reagent SABC (Beyotime, Shanghai, China) were used for coating the primary antibody. Finally, the MCP-1 and Sirt1-positive cells were exposed to diaminobenzidine (DAB; Solarbio, Beijing, China).

Immunofluorescence (IF) Analysis for c-Fos and c-Jun Activation

The AP-1 activation is mainly achieved by phosphorylation of c-Fos and c-Jun in the cell nucleus. After treatments, the NP cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X and blocked with 5% bovine serum albumin (BSA) for the preparation of IF staining. Following, the cells were incubated with phosphor-c-Fos (ab27793, Abcam, Cambridge, MA, USA) or c-Jun (ab32385, Abcam, Cambridge, MA, USA) primary antibody overnight at 4°C. The next day, NP cells were continuously incubated with Alexa Fluor488 secondary antibody (Invitrogen, Carlsbad, CA, USA) at room temperature. The staining intensity of the fluorescence was represented the expression of phosphorylated c-Fos and c-Jun.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNA from the NP cells was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), which was then reverse-transcribed into complementary deoxyribose nucleic acid (cDNA) for qPCR according to the manufacturer's instructions following the system as 30°C for 10 min, 42°C for 20 min, and 95°C for 4 min. Then, qPCR amplification was performed with the qPCR SYBR Green Master kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions following the system as 95°C for 4 min, 30 cycles of 95°C for 45 sec, 62°C for 45 sec, 72°C for 60 sec, 95°C for 1 sec, and kept at 4°C. All relative mRNA expression was achieved by normalization to the endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH), according to the $2^{-\Delta\Delta Ct}$ method. The primers used are listed as follows: Sirt1-forward (f), 5'-TAGCCTTGTCAGATAAGGAAGGA-3'; Sirt1-reverse (r), 5'-ACAGCTTCACAGTCAACTTTGT-3'; MCP-1-f, 5'-AGAATCACCAGCAGCAAGTGTCC-3'; MCP-1-r, 5'-TCCTGAACCCACTTCTGCTTGG-3'; type II collagen-f, 5'-TGGACGATCAGGCGAAACC-3'; type II collagen-r, 5'-GCTGCGGATGCTCTCAATCT-3'; GAPDH-f, 5'-ACAACTTTGGTATCGTGGAAGG-3'; GAPDH-r, 5'-GCCATCACGCCACAGTTTC-3'.

Cell Viability Assay

We determined the effect of the drug treatments on the cell viability by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Kumamoto, Japan). The cells were seeded in 96-well plates with a density of 5000/well and incubated with the indicated drugs. Then, NP cells were incubated with CCK-8 reagent according to the manufacturer's instructions. The absorbance was determined at 450 nm wavelength using a microplate reader. Cell viability was shown as a percentage relative to non-treated value. All tests were performed in triplicate.

Statistical Analysis

Data analysis was performed using GraphPad Version 6.0 (San Diego, La Jolla, CA, USA), and the results were expressed as mean ± standard deviation (SD). The differences between the two groups were analyzed by using the Student's *t*-test. A comparison between multiple groups was made using one-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ were considered to be statistically significant between groups.

Results

The MCP-1 and Sirt1 Expression in Human NP Tissues

To elucidate whether the expression MCP-1 and Sirt1 were different between the degenerate

and the non-degenerate intervertebral disc, we used the IHC method to determine the protein level of MCP-1 and Sirt1 in the NP tissues. As shown in Figure 1A, the NP cells stained with the brown-yellow substances were deemed to be positive. The MCP-1 positive cells of the IDD condition was significantly higher than the control. By contrast, the Sirt1 protein expression was reduced when the disc degenerated (Figure 1B). Therefore, the expression of MCP-1 and Sirt1 was negatively related during the progression of IDD.

The MCP-1 and Sirt1 Expression in IL-1β Treated NP Cells In Vitro

To clear the relation between MCP-1 and Sirt1 expression *in vitro*, we used IL-1β to induce the NP cell degeneration. NP cells were cultured with three different concentrations from 5, 10 to 20 ng/mL for three days. The expression of the type II collagen was used as a marker to value the degenerative degree of NP cells. Compared to the control without treatment, the type II collagen mRNA of NP cells significantly decreased when the dose of IL-1β was higher to 10 ng/mL, which was more evident in the 20 ng/mL group (Figure 2A). However, the MCP-1 mRNA expression was gradually reduced with dose-dependence from 5 ng/mL IL-1β compared to the control (Figure 2B). Additionally, the Sirt1 expression was also suppressed when the degree of NP cell degeneration deepened (Figure 2C). As the results *in vivo*, the expression of MCP-1 and Sirt1 was also negatively related during the development of NP cells degeneration *in vitro*.

Sirt1 Regulates MCP-1 Expression During NP Cells Degeneration In Vitro

10 ng/mL IL-1β in the culture medium was used for inducing NP cell degeneration in the following experiments. To determine whether the changed Sirt1 also affected MCP-1 expression, we regulated Sirt1 expression with Nico (100 μM) and Res (50 μM) and suppressed MCP-1 by Bin (75 μM). The NP cells were cultured with IL-1β only (as a normal group), combined with Nico, Res, or Bin for three days. As shown in Figure 3A, Nico significantly suppressed the Sirt1 mRNA expression, and Res increased Sirt1

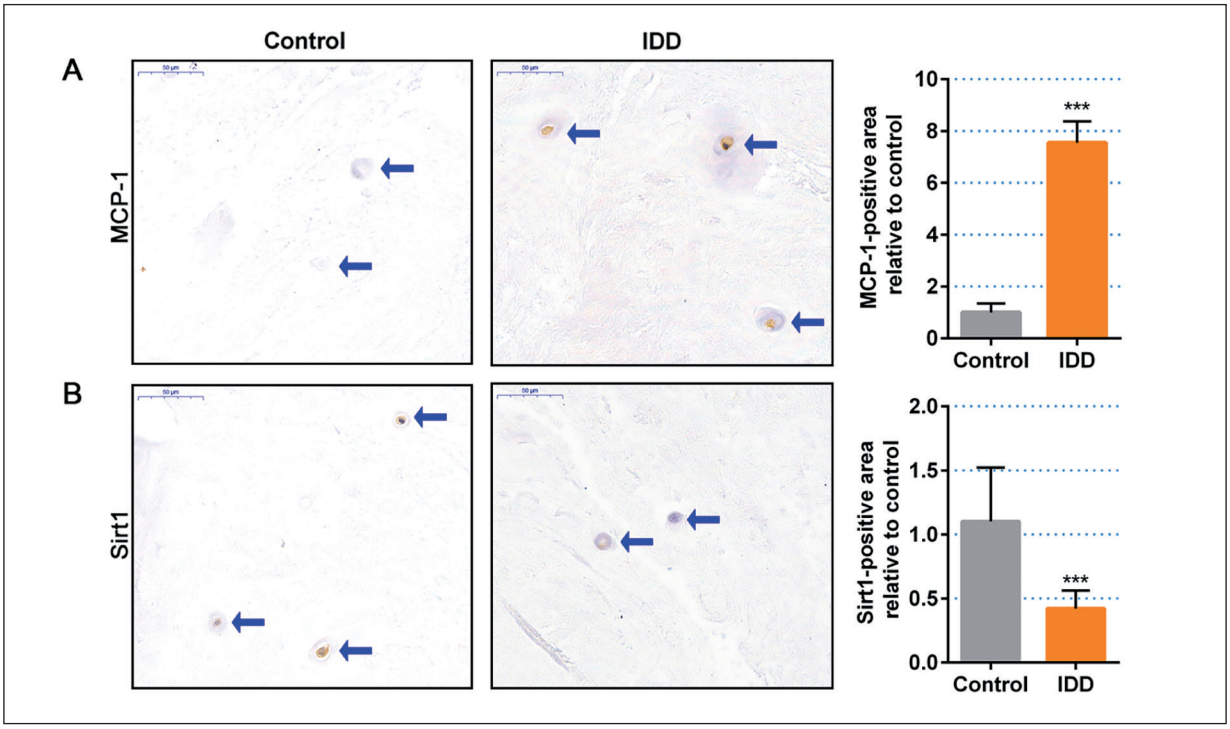

Figure 1. The MCP-1 and Sirt1 expression in human NP tissues. The NP collected from the spinal trauma patients without degeneration was grouped as control. The NP received from the disc herniated patients was arranged as the IDD. **A,** IHC staining of MCP-1 and the corresponding quantification analysis (magnification: 400×). **B,** IHC staining of Sirt1, and the corresponding quantification analysis (magnification: 400×). Data as mean ± SD versus the control group. Statistically significant differences versus control group *** less than 0.001 are marked.

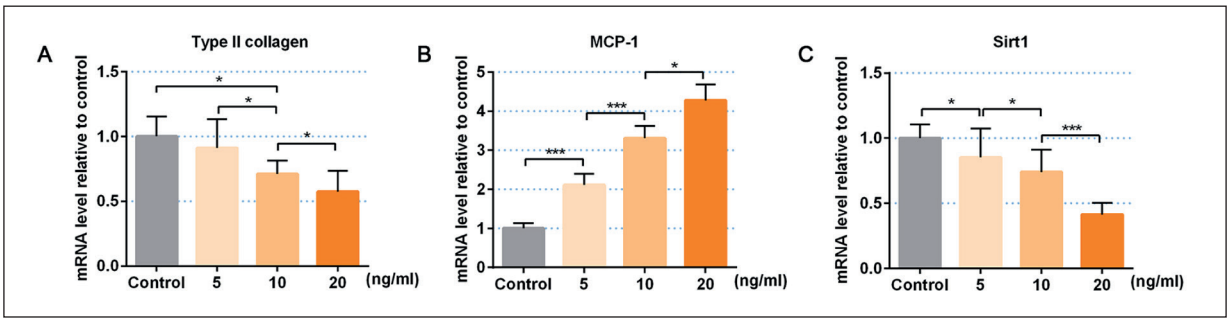

Figure 2. The MCP-1 and Sirt1 expression in IL-1β treated NP cells. The NP cells without any treatments were grouped as control. The m RNA expression of (**A**) type II collagen, (**B**) MCP-1, and (**C**) Sirt1 was determined by RT-qPCR. Data as mean ± SD. Statistically significant difference * less than 0.05, or *** less than 0.001 are marked.

expression compared to the normal group. The suppression of MCP-1 by Bin did not affect Sirt1 expression compared to the normal group (Figure 3A). Besides, the downregulation of Sirt1 further promoted the MCP-1 expression compared to the control and normal group, and the upregulation of Sirt1 reduced the MCP-1 expression, as well as Bin treatment compared to the normal group (Figure 3B). Therefore, the Sirt1 upregulation led to the suppression of MCP-1, and the down-regulation of Sirt1 in the degenerated NP cells resulted in the further increase of MCP-1 in the NP cells. Form the result of the type II collagen expression (Figure 3C) and CCK-8 (Figure 3D)

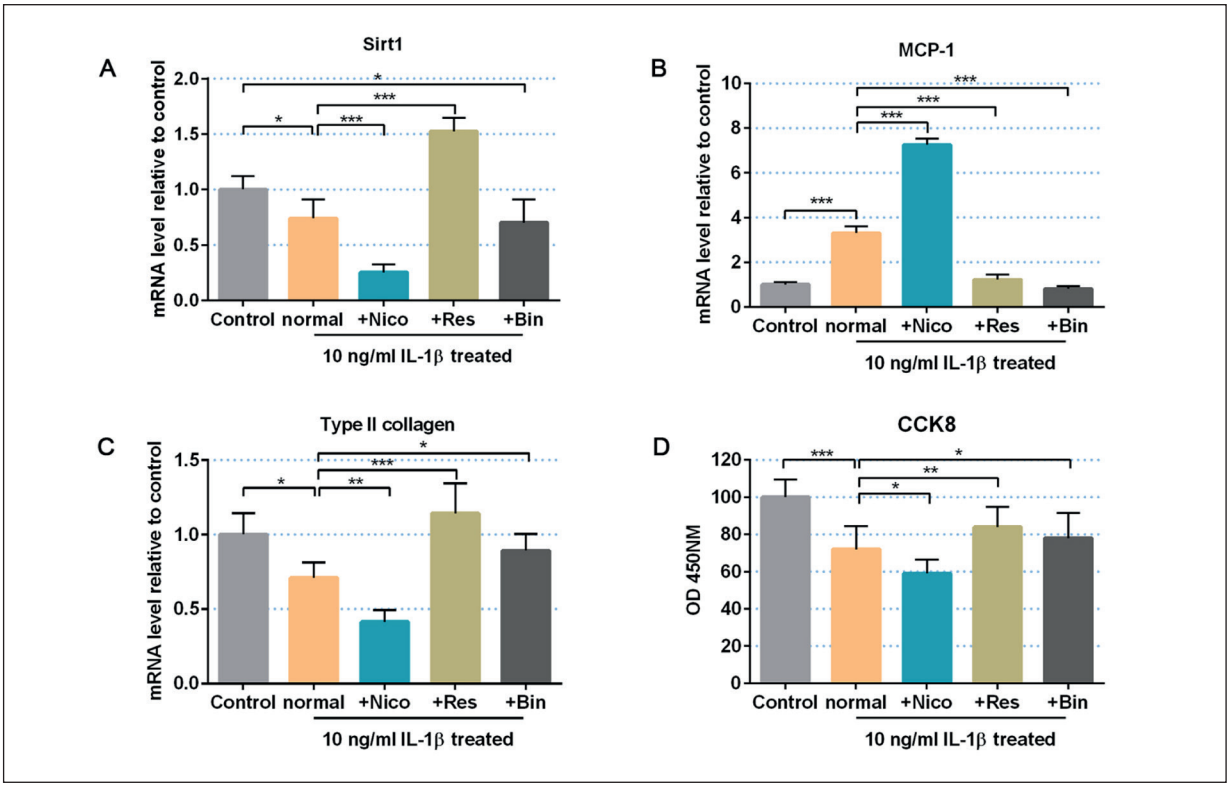

Figure 3. Sirt1 regulates MCP-1 expression in IL-1β treated NP cells. The NP cells without any treatments were grouped as control. The NP cells incubated with IL-1β only were arranged as normal. The m RNA expression of (**A**) Sirt1, (**B**) MCP-1, and (**C**) type II collagen was revealed by RT-qPCR. **D,** NP cells viability was determined by CCK8 assay. Data as mean ± SD. Statistically significant difference * less than 0.05, ** less than 0.01, or *** less than 0.001 are marked.

analysis, we knew that inhibiting MCP-1 expression could prevent NP cells from the IL-1β which caused an injury that maintained the cell function and viability.

Sirt1 Regulates c-Fos and c-Jun Phosphorylation

To determine the activation of AP-1, we tested the phosphorylation of its subunits c-Fos and c-Jun under each treatment. After receiving the physical and chemical stimulus, the phosphorylated c-Fos and c-Jun accumulate mainly in the cell nucleus. In contrast with the control, the phosphorylated c-Fos (pc-Fos) was significantly increased after IL-1β treatment, which was enhanced by the suppression of Sirt1 or weakened by upregulating of Sirt1 compared to the normal group. However, the destruction of MCP-1 did not inhibit pc-Fos (Figure 4A). Apart from this, the phosphorylated c-Jun (pc-Jun) was also raised after NP cells degeneration caused by IL-1β. Nico suppressed Sirt1 expression aggravat-

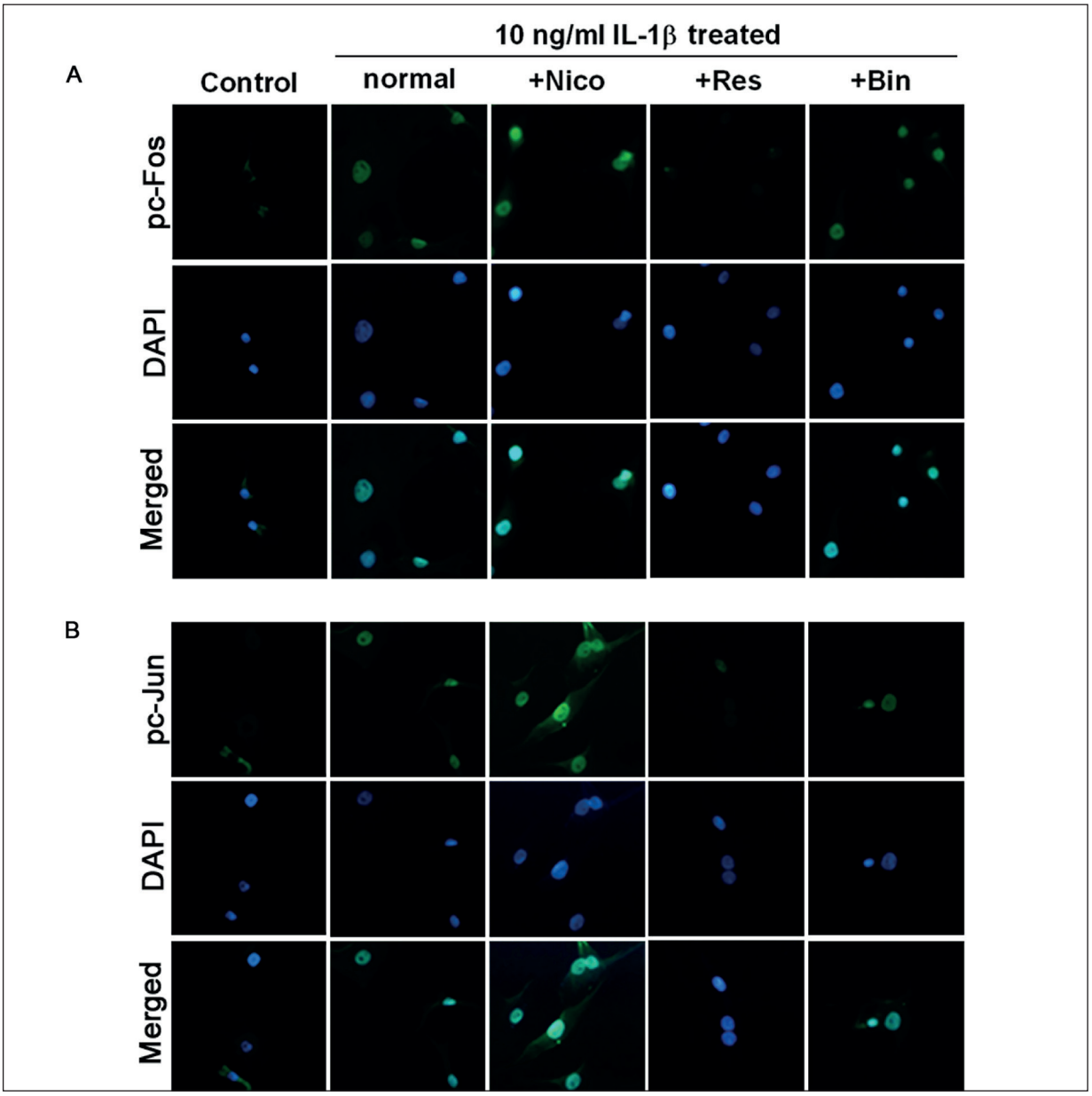

Figure 4. Sirt1 regulates c-Fos and c-Jun phosphorylation. **A,** IF staining: Green indicates the phosphorylated c-Fos, blue indicates the cell nucleus (magnification: 200×). **B,** IF staining: Green indicates the phosphorylated c-Jun, blue indicates the cell nucleus. (magnification: 200×).

ing the accumulation of pc-Jun, and Res inhibited the pc-Jun in the nucleus. The pc-Jun level did not significantly change with the presence of Bin compared to the normal group (Figure 4B). Therefore, the activation of AP-1 was regulated by the Sirt1 expression but not affected by the MCP-1 level.

AP-1 Inactivation Suppresses MCP-1 Expression

The above results indicated the AP-1 activation accompanied by the upregulation of MCP-1 and the inactivation of AP-1 by Sirt1 attended with the downregulation of MCP-1. To clarify whether the suppression of AP-1 led to the decreased MCP-1, we used the inactivator of AP-1 (10 μM) and tested the mRNA expression of MCP-1. As shown in Figure 5A, under the treatment of 10 ng/mL IL-1β, the SR suppressed the pc-Fos and pc-Jun expression in the cell nucleus compared to the normal group. Besides, SR suppressed the MCP-1 mRNA but did not affect the Sirt1 expression compared to the normal. After the suppression of AP-1 by SR, the type II collagen and cell viability

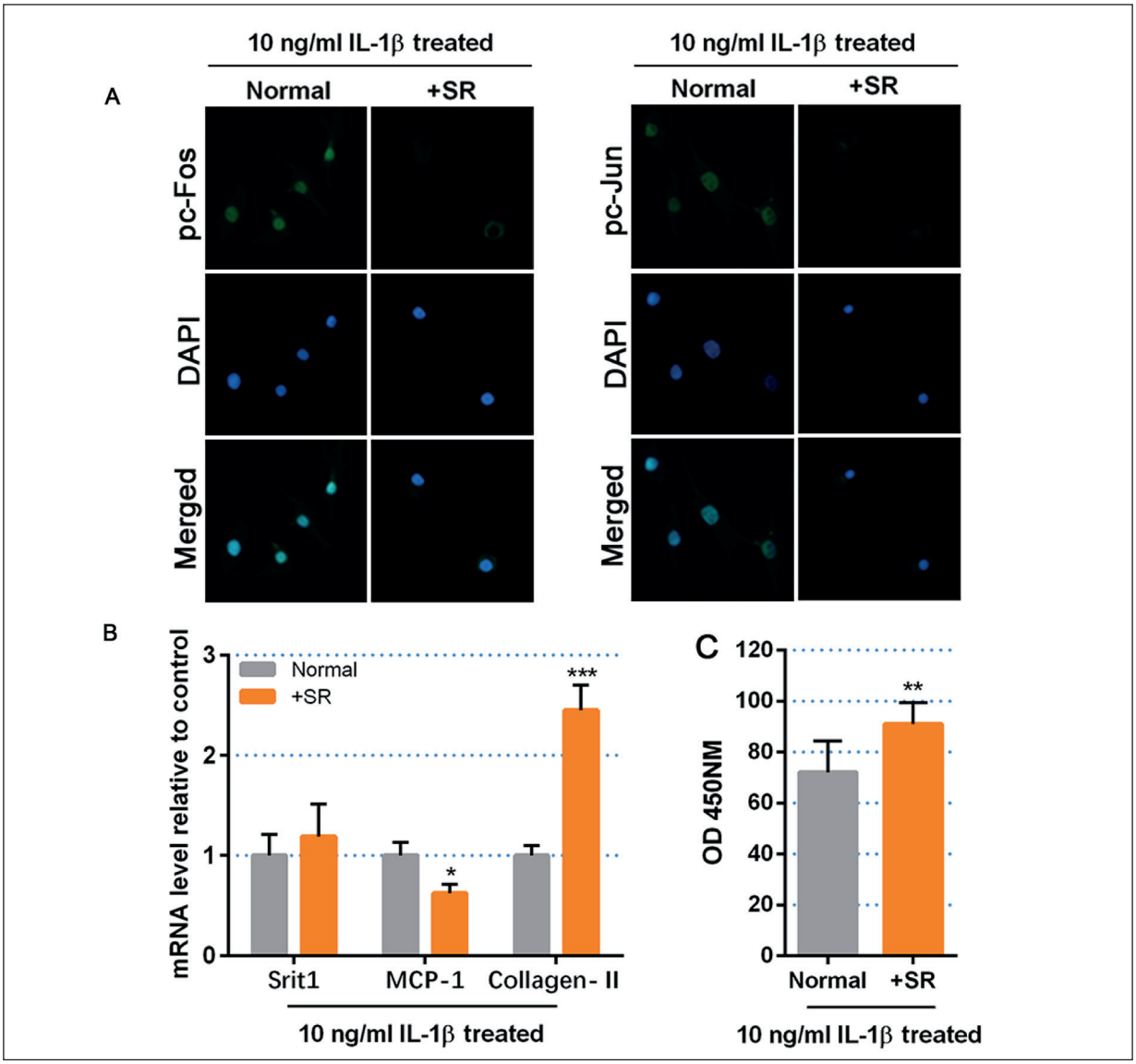

Figure 5. AP-1 inactivation suppresses MCP-1 expression. The NP cells were treated with IL-1β with or without SR. **A,** IF staining: Green indicates the phosphorylated c-Fos or c-Jun, blue indicates the cell nucleus (magnification: 200×). **B,** The m RNA expression of Sirt1, MCP-1, and type II collagen was determined by RT-qPCR. **C,** NP cell's viability was determined by CCK-8 assay. Data as mean ± SD. Statistically significant difference * less than 0.05, ** less than 0.01, or *** less than 0.001 are marked.

were also improved (Figure 5B, 5C). These results indicated that the MCP-1 was a downstream gene regulated by the activity of AP-1.

Discussion

The intervertebral disc is composed of the central NP, the peripheral annulus fibrosus, and the cartilage endplates. The NP is significant for its overall structure and mechanical conduction function[24]. Therefore, most of the research on IDD focus on the study of the NP. In the degenerated NP tissues, we found that MCP-1 overexpressed NP cells, and MCP-1 also showed a dose-dependent increase in the IL-1β treated NP cells *in vitro*. MCP-1 is an inducer of the inflammatory factors and can promote the process of inflammatory response. The activated MCP-1 recruits macrophages to the joints aggravating the inflammation in the pathological process of rheumatoid arthritis[25]. MCP-1 is also considered to be related to the muscle nerve pain through its cognate receptor[26]. NP cells can produce MCP-1 under the stimulation of inflammatory factors, and the expression of MCP-1 is positively correlated with the severity of IDD[9,27]. Thereby, the suppression of the entire inflammatory response process by inhibiting MCP-1 may be a potential treatment for discogenic LBP.

In the experiments, we found that the expression of Sirt1 in NP cells was opposite to the trend of MCP-1 both *in vivo* and *in vitro*, which shows that SIRT 1 and MCP-1 expression may have a negative correlation. Res is a class of polyphenols, the Sirt1 agonist, with a variety of biological activities, including anti-inflammation. Nico, a water-soluble Vit B3, is an inhibitor of Sirt1. The overexpression of Sirt1 suppressed the MCP-1 expression in IL-1β treated NP cells. When Sirt1 was further inhibited, the increase in MCP-1 expression aggravated. Therefore, Sirt1 has the effect of downregulating MCP-1 in the degenerative NP cells.

As a nuclear transcription factor, AP-1 is sufficient to regulate the inflammatory response. In the CHs, AP-1 inhibits the production of inflammatory factors and control the progress of arthritis[28]. AP-1 regulates the expression of MCP-1 in a variety of cells. In airway smooth muscle, AP-1 promotes MCP-1 expression and aggravates inflammation[29]. In brain endothelial cells, AP-1 can also mediate the activation of MCP-1[30]. Among the many AP-1 dimers, the AP-1 heterodimer formed by Fos and Jun is the most stable. Commonly, c-Jun is not expressed in cells or has only a shallow level of expression[31]. When stimulated by extracellular physicochemical factors or intracellular signaling cascades, c-Jun is activated by phosphorylation into the nucleus and forms a heterodimer with the pc-Fos, which is also accumulated in the nucleus[31]. Therefore, the expression levels of pc-Fos and pc-Jun in the nucleus can indicate the activity level of AP-1.

In order to clarify the relationship between AP-1 and MCP-1 in the degeneration process of NP cells, we added AP-1 inhibitor SR in the IL-1β treated NP cells and found that SR also inhibited the MCP-1 expression, which indicates that AP-1 involves the IL-1β-triggered MCP-1 upregulation in NP cells. Furthermore, the inhibitory function of Sirt1 on the activation of c-Fos and c-Jun subunits has also been confirmed in some studies[32,33]. To determine whether Sirt1 can inhibit the activation of AP-1 subunits in the degenerated NP cells, we regulated Sirt1 expression through its agonists and inhibitors. We found that Sirt1 activity was negatively correlated with pc-Fos and pc-Jun. Based on the above results, we speculate that Sirt1 can inactivate c-Fos/c-Jun, and then, downregulate the downstream MCP-1.

Conclusions

In summary, Sirt1 can suppress the MCP-1 production in the IL-1β induced NP cells degeneration, which may be related to the deficient activity of the AP-1 subunits c-Fos/c-Jun. The novelty of this present study is to elucidate the anti-inflammatory effect of Sirt1 during IDD is also related to the AP-1 mediated MCP-1 suppression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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