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Low-Frequency Cyclic Stretch Upregulates the Expression of Nuclear Factor Erythroid 2-Related Factor 2 in Human Nucleus Pulposus Cells to Inhibit the Resistin-Induced Interleukin-20 Expression

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ABSTRACT

Background: Resistin may connect obesity and intervertebral disc (IVD) degeneration (IDD) and is linked with chronic inflammation. Furthermore, human IDD is characterized by high expression of interleukin-20 (IL-20). The response of human nucleus pulposus (NP) cells to tensile forces depends on both the duration and magnitude of the force applied. Nonetheless, the interactions among resistin, IL-20, and tensile forces in regulating the function of IVD NP cells remain yet to be fully understood. Nuclear factor erythroid 2-related factor 2 (NRF2) has been identified as a pleiotropic protein that enhances cellular resistance to stress stimuli and inflammatory challenges. The role of NRF2 in NP cells is not well defined, and whether tensile force influences NRF2 levels in NP cells is not known.

Aims: This study investigated the role of NRF2 in human NP cells subjected to low-frequency cyclic stretch stimulation, and the underlying mechanisms involved.

Materials and Methods: Human NP cells were cultured in chambers, serum-starved for 12 h, then subjected to 5% cyclic strain at 0.1 Hz in a bioreactor. Control chambers had no stretching. Cells were lysed for analysis post-loading.

Results: Resistin stimulation induced the expression of IL-20 in human NP cells in a dose- and time-dependent manner. The activation of the p38 mitogen-activated protein kinase, and Akt signaling pathways, as well as the production of toll-like receptor 4, are necessary to render resistin to cause the release of IL-20. In NP cells, transcription factor enzyme-linked immunosorbent assays revealed that resistin led to an increase in nuclear factor (NF)-κB-DNA binding activities. Exposure of NP cells to 5% cyclic stretch at 0.1 Hz inhibited this resistin-induced NF-κB activation and IL-20 expression.

Discussion: These findings elucidate the molecular mechanisms through which resistin induces IL-20 expression in NP cells and also demonstrate that low-frequency cyclic stretch can protect against this induction.

Chia-Kung Yen and Hung-Yu Pan contributed equally to this work.

1 | Introduction

In the spinal milieu, cells within the intervertebral disc (IVD) experience persistent mechanical stimuli, impacting their biochemical and physical characteristics crucial for sustaining disc functionality [1]. The outer annulus fibrosis functions as a robust, ligament-like structure shielding the inner nucleus pulposus (NP), which acts as a semifluid cushion, reducing the longitudinal pressure applied to the body [2]. With advancing age, IVD can undergo degenerative changes due to factors such as poor posture, excessive mechanical stress, or natural wear and tear. This can compromise IVD function, hinting at the potential involvement of mechanical forces in the degenerative processes affecting the disc [3]. NPcells endure mechanical loading and sustained compressive forces during daily movements, which has been shown to cause alterations in gene expression and cellular activity that contribute to counteracting disc degeneration [2, 4]. Moreover, under heightened mechanical loading, NP cells demonstrate anti-inflammatory properties [5]. This mechanism sheds light on the potential antidegenerative effects of these cells on IVD health and pathology.

Interleukin (IL)-20, a cytokine integral to inflammatory responses and tissue repair, plays a significant role in IVD degeneration (IDD) [6]. Our previous study demonstrated that IL-20 can enhance inflammatory responses in IVD, thereby influencing the progression of disc degeneration and associated pathological changes [7]. IL-20 exerts its effects on target cells primarily through its receptors, such as IL-20R1 and IL-20R2, impacting cellular functions, including proliferation, differentiation, and migration [8, 9]. IL-20 contributes to disc degeneration through complex and multifaceted mechanisms. Extracellular factors originating from inflammatory or injured tissues can modulate IL-20 expression [7, 10]. However, the associated molecular mechanisms involved in enhanced IL-20 expression under pathophysiological conditions remain poorly understood. The role of IL-20 in IDD needs to be investigated further to clarify the relationship between IL-20 and disc degeneration, as well as to identify potential therapeutic targets for treating related diseases.

Resistin, a hormone secreted by adipocytes, is implicated in various metabolic disorders, including obesity, diabetes, and cardiovascular diseases [11], and may also have a significant role in the pathogenesis of IDD [12]. The capacity of resistin to modulate inflammatory responses is a pivotal pathway linking it to IDD [13]. Furthermore, resistin can stimulate the secretion of inflammatory mediators such as tumor necrosis factor-α and IL-1β [14]. These cytokines have a key role in driving degenerative changes within IVD, thereby accelerating structural deterioration and functional impairment of disc tissues [15]. These findings help us understand how resistin potentially affects IDD, but still do not fully explain how resistin affects the expression of IL-20 or how mechanical stretching affects the expression of genes that are affected by resistin. Hence, these molecular mechanisms require further research to fully elucidate and definitively confirm these associations.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a key transcription factor that orchestrates cellular antioxidant defenses and stress response pathways [16]. Besides its role in redox homeostasis, NRF2 substantially contributes to anti-inflammatory processes. It regulates inflammatory responses through several mechanisms, such as promoting antioxidant effects, suppressing the expression of inflammatory genes, and interacting with the nuclear factor (NF)-kB pathway [17]. In this study, the interplay between cyclic stretching and resistin stimulation was examined in the context of regulating cytokine gene expression in NP cells. Our findings elucidate that resistin-mediated induction of IL-20 expression occurs through the activation of Toll-like receptor 4 (TLR4), p38 MAPK, and Akt, and the downstream transcription factor NF-κB. In addition, the exposure of NP cells to short-term low-frequency cyclic stretching could attenuate resistin-induced IL-20 mRNA expression through upregulation of NFR2. These results illuminate the mechanism through which mechanical forces suppress resistin-induced responses in NP cells and offer novel insights into the pathophysiology underlying the modulation of disc degeneration.

2 | Methods

2.1 | Reagents

Recombination human resistin was purchased from PeproTech (Rocky Hill, NJ, USA) and dissolved in deionized water to prepare a stock solution of 50 μg/mL. PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), LY294002 (PI3K/Akt inhibitor), and SN50 (NF-κB p65 inhibitor) were purchased from Sigma (St. Louis, MO, USA). The secondary antibody against rabbit IgG, NRF2-, phospho-p38-, p38-, phospho-Akt-, Akt-, and TLR4-specific antibodies (rabbit polyclone) were bought from Cell Signaling Technology (Beverly, MA, USA). All culture materials were purchased from Gibco (Grand Island, NY, USA).

2.2 | Culture of NP Cell Lines

Human NP cells were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in a complete growth medium containing 10% fetal bovine serum. Cells at passages 3–6 were used for assays. Once the cells reached 80% confluence, they were trypsinized and seeded into stretching chambers.

2.3 | Cyclic Stretching

Commercial stretching chambers (Taihoya, Taiwan) were coated overnight with 50 μg/mL fibronectin (FC010, Merck Millipore, Darmstadt, Germany) at 37°C. Human NP cells were then seeded in these chambers (10×10^4 cells per chamber in 5 mL of growth medium) and cultured for 72 h at 37°C in a 5% CO_2 chamber, unless otherwise specified. To synchronize the cells and reduce the influence of serum-derived growth factors, the cells were maintained for 12 h in a serum-free medium for serum starvation. The chambers were then placed on a commercial stretching bioreactor (ATMS BOXER, Taihoya, Taiwan) and subjected to a 5% cyclic sinusoidal uniaxial strain at a frequency of 0.1 Hz at 37°C and 5% CO_2 [5]. Control chambers were maintained under

identical conditions with no stretching. Cells were stretched for varying durations. Stretching was performed in the designated chambers for 24 h and progressively included those designated for shorter durations so that all stretching ended simultaneously. The cells were lysed immediately following the mechanical loading for further analysis [18].

2.4 | Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted and reverse transcription was performed as previously described. Gene expression was measured by quantitative real-time PCR on an ABI Prism 7900HT system (CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). PCR products were subjected to melting curve analysis, and the data were evaluated using the $2^{-\Delta\Delta CT}$ method and normalized to 18S rRNA and to the control NP cells [19]. The specific primer sequences used for the real-time PCR were IL-20 (forward: 5′-AGA TCA GCA GCC TCG CCA ATT C-3′; reverse: 5′-CAA AGT GAC TCA GAA TCT GGC TG-3′) and 18S rRNA (forward: 5′-CGG CGA CCC ATT CGA AC-3′; reverse: 5′-GAA TCG AAC CCT GAT TCC CCG TC-3′).

2.5 | IL-20 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-20 in the media were measured by a sandwich ELISA kit (R & D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

2.6 | Nuclear and Cytosolic Fractionation

Following collection, NP cells were washed twice with phosphate-buffered saline (PBS) and then subjected to nuclear and cytoplasmic fractionation using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's protocol. Each isolated protein fraction was subsequently analyzed via Western blot analysis.

2.7 | Western Blot Analysis

Cells were lysed with a buffer containing NP-40 (1%), sodium deoxycholate (0.5%), SDS (0.1%), and a protease inhibitor cocktail (including phenylmethylsulfonyl fluoride, aprotinin, and sodium orthovanadate). A total of 50 μg of protein from the cell lysate was separated using SDS-polyacrylamide gel electrophoresis using a 12% resolving gel and a 4% stacking gel. The separated proteins were then analyzed using the specified antibodies and the Western-Light chemiluminescent detection system (Bio-Rad, Hercules, CA, USA), as detailed previously [19].

2.8 | siRNA Transfection

The gene-specific siRNAs, including control (sc-37007), p65 (sc-29410), TLR4 (sc-40260), and NRF2 (sc-37030), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For siRNA transfection, cells were transfected with the specified construct using the RNAiMAX transfection kit (Invitrogen, Carlsbad, CA, USA). Human NP cells (80% confluence) were cultured for 24 h and then transfected with the indicated siRNA (100 nM) against p65, TLR4, and NRF2, which could knockdown ~80% expression of the corresponding gene compared to the control siRNA-transfected cells (data not shown).

2.9 | NF-kB p65 Activity Assay

Nuclear protein extracts were isolated and collected. NF-κB p65 transcriptional activity was assessed by measuring its binding to specific DNA sequences using the NF-κB (p65) Transcription Factor Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Equal amounts of nuclear protein from each experimental condition were analyzed according to the manufacturer's protocol. Sample absorbance was measured at 450 nm using a microplate reader.

2.10 | Statistical Analysis

The results presented in this study are expressed as the mean ± standard error of the mean. Statistical analysis was conducted using an independent Student's *t*-test for two-group comparisons and an analysis of variance followed by Scheffé's test for multiple comparisons. A p value of < 0.05 was considered statistically significant.

3 | Results

3.1 | Resistin Treatment Induces the Upregulation of IL-20 in Human NP Cells

The effects of resistin on IL-20 expression in human NP cells were evaluated in vitro. The time courses determined for the IL-20 mRNA levels demonstrated an increase after 1 h of 50 ng/mL resistin stimulation and a peak expression at 2 h, followed by a gradual reduction at 12 h thereafter (Figure 1A). Resistin exposure of NP cells caused significant increase in the IL-20 secretion levels in the conditioned medium (Figure 1C) in a time-dependent manner. Figure 1B,D shows that resistin dose dependently induces IL-20 mRNA expression and IL-20 secretion, respectively, in the conditioned medium in NP cells.

3.2 | Resistin-Induced IL-20 Expression in NP Cells Is Mediated by the p38 MAPK and Akt Signaling Pathways

The MAPK and phosphoinositide 3-kinases (PI3K)/Akt pathways are known to modulate genes expressing inflammatory factors in IVD cells [20, 21]. NP cells were pretreated with specific inhibitors of extracellular signal-regulated kinase (ERK; PD98059; 30 μM), c-JUN N-terminal kinase (JNK; SP600125; 20 μM), p38 (SB203580; 10 μM), or PI3K/Akt (LY294002; 30 μM) for 1 h before and during 50 ng/mL resistin administration to determine any possible role of MAPK- or PI3K/Akt-dependent pathways in resistin-induced

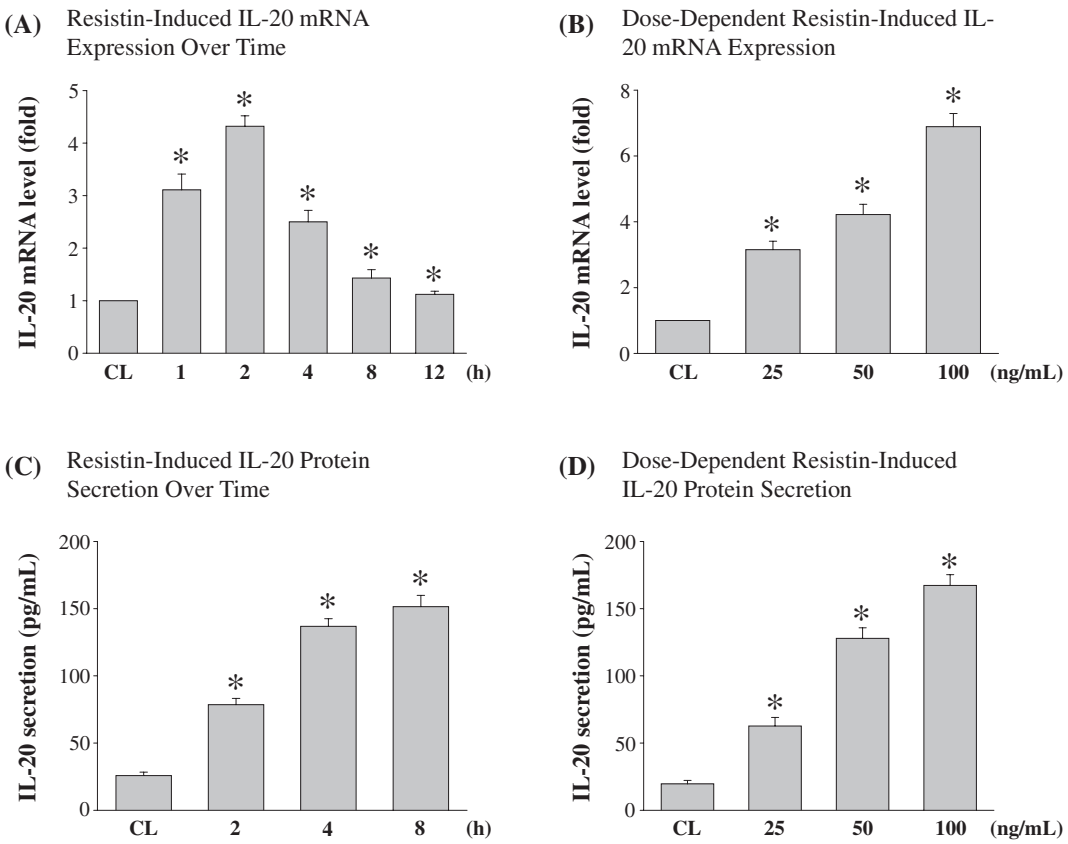

FIGURE 1 | Induction of IL-20 expression in NP cells by resistin stimulation. RNA samples were isolated at the indicated time points or doses and subjected to real-time polymerase chain reaction analysis. Data are presented as fold changes in fluorescent density from control NP cells (CL) normalized to 18S rRNA level (A, B). The IL-20 protein in conditioned media was detected by enzyme-linked immunosorbent assay (C, D). NP cells were kept as controls (CL) or stimulated with 50 ng/mL resistin for the times indicated (C), or the cells were stimulated with resistin at various doses for 4 h (D). Data are shown as mean ± standard error of the mean (SEM). *$p < 0.05$ versus control NP cells (CL).

IL-20 expression. A significant inhibition of resistin-induced IL-20 mRNA expression (Figure 2A) and secretion (Figure 2B) in NP cells was observed upon treating the cells with SB203580 and LY294002, but not with PD98059 and SP600125. After 50 ng/mL resistin stimulation, the phosphorylation of p38 MAPK and Akt (Figure 2C) in NP cells increased rapidly, reaching maximal levels at 30 min. After such transient increases, the levels of phosphorylation decreased after 2 h of treatment.

3.3 | NF-kB Is a Major Determinant of the Resistin Induction of IL-20 Expression

The human IL-20 gene promoter harbors multiple binding sites for transcription factors, including NF-kB [22]. We assessed the involvement of NF-kB activation in the signal transduction pathway leading to resistin-induced IL-20 mRNA expression. Human NP cells were incubated with SN50 (50 μM), a specific inhibitor for NF-kB for 1 h or transfected with p65 siRNA, followed by stimulation with resistin for 2 h. The resistin-induced IL-20 mRNA expression in NP cells was significantly reduced through inhibition with SN50 or p65 siRNA, indicating that NF-kB is the major transcription factor involved in the regulation of IL-20 gene induction (Figure 3A). To investigate any potential binding of NF-kB with the IL-20 promoter region in NP cells, we quantitatively analyzed the in vitro NF-kB p65 binding activity using ELISA kits for transcription factors. When resistin was added to NP cells, NF-kB p65-binding DNA activity increased after 0.5 h and stayed high for at least 2 h (Figure 3B).

To evaluate whether the inhibition of IL-20 expression through the p38 MAPK and Akt signaling pathways occurs at the transcriptional level, we examined the effects of specific inhibitors targeting p38 and Akt upon resistin-induced NF-kB p65 activity. Culturing of the NP cells in the presence of resistin increased the activity of NF-kB p65 compared with unstimulated cells upon normalization with a transfection control (Figure 3C). Pretreatment of the cells with SB203580 and LY294002 led to a marked inhibition of the resistin-induced NF-kB p65 activation.

3.4 | Blockade of TLR4 Inhibits Resistin-Induced IL-20 Expression and NF-κB Activation

TLR4 serves as a cell surface receptor for resistin in several human cell types [23–25]. We investigated the role of TLR4 in resistin-induced IL-20 expression in NP cells by evaluating the effects of neutralizing polyclonal anti-TLR4 antibody (20 μg/mL) and TLR4-siRNA on resistin-induced IL-20 mRNA expression and p65 activation. Resistin-induced IL-20 mRNA expression was significantly inhibited upon the incubation of NP cells with siRNA or a neutralizing antibody of TLR4 (Figure 4A). In addition, NP

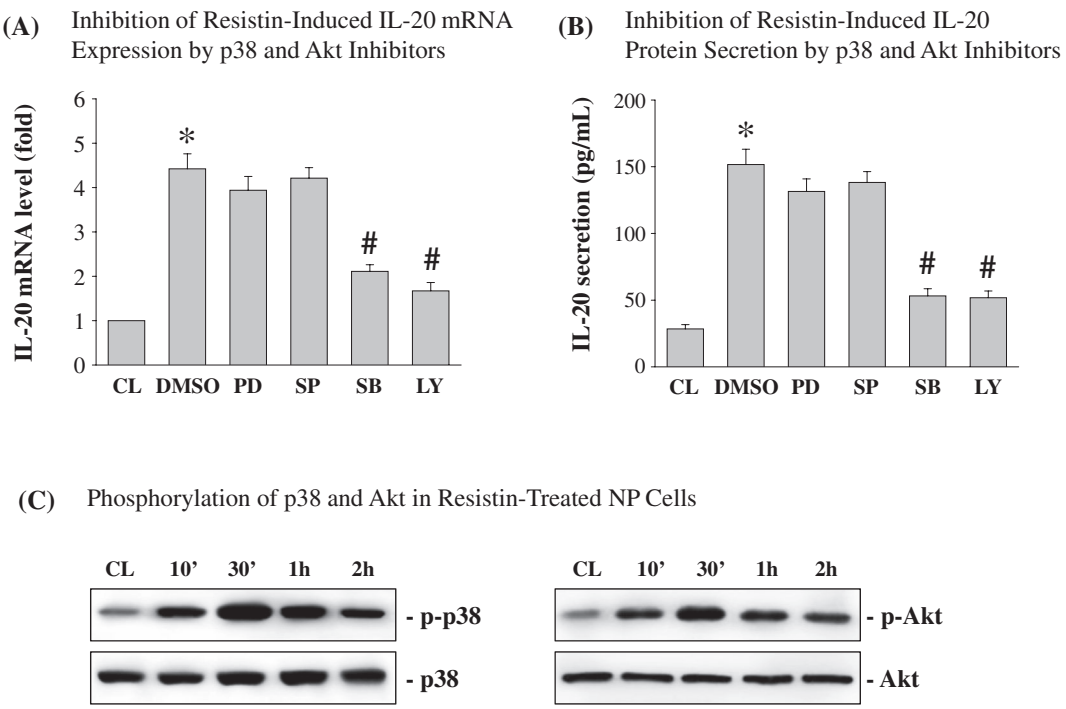

FIGURE 2 | p38 MAPK and Akt pathways are required for resistin-induced expression of IL-20. (A, B) NP cells were kept as control (CL) or stimulated with 50 ng/mL resistin for 2 h (A) and 4 h (B). Before being kept as CL or stimulated with resistin, NP cells were pretreated with PD98059 (PD), SP600125 (SP), SB203580 (SB), or LY294002 (LY) individually for 1 h. (A) RNA was isolated and subjected to real-time polymerase chain reaction analysis. Data are presented as fold changes in fluorescent density from control NP cells (CL) normalized to 18S rRNA level. (B) The IL-20 protein secretion in conditioned media was determined by enzyme-linked immunosorbent assay. The results are shown as mean ± standard error of the mean. *$p < 0.05$ versus CL. #$p < 0.05$ versus vehicle control (dimethyl sulfoxide) with resistin stimulation. (C) NP cells were employed as CL or stimulated with resistin for the durations indicated, and p38 and Akt phosphorylations were determined by Western blotting.

cells pretreated with a neutralizing antibody for TLR4 also caused a significant reduction in NF-kB p65 activation (Figure 4B).

3.5 | Exposure of NP Cells to Cyclic Stretch of 5% and 0.1 Hz Inhibits Resistin-Induced IL-20 Expression

Under static conditions, stimulation of NP cells with resistin increased IL-20 expression (Figure 5A). Pre-exposure of NP cells to a cyclic stretch of 5% with 0.1 Hz for 30 min before resistin stimulation significantly inhibited resistin-induced IL-20 mRNA expression. However, the same inhibitory effect was not noted on pre-exposing NP cells to a cyclic stretch of 5% with 0.1 Hz for 2 h (Figure 5A). When NP cells were exposed to a cyclic stretch of 5% with 0.1 Hz for 30 min, p38 MAPK and Akt phosphorylation was greatly reduced (Figure 5B). Additionally, pre-exposure of NP cells to 5% cyclic stretch at 0.1 Hz for 30 min led to a reduction in resistin-induced p65 NF-kB-DNA-binding activity (Figure 5C).

3.6 | Exposure of NP Cells to Cyclic Stretch of 5% and 0.1 Hz Activates the NRF2 to Attenuate the Resistin Effect on the IL-20 Expression in These Cells

The activation of NRF2 attenuates proinflammatory transcription factor activation and cytokine expression [16]. Exposure of NP cells to 5% cyclic stretch at 0.1 Hz for 30 min resulted in marked activation of NRF2 in the nucleus (Figure 6A). Moreover, treatment of NP cells with NRF2-specific siRNA to knock down the expression of respective genes expression significantly restored the effect of 5% cyclic stretch at 0.1 Hz on lowering the resistin-induced IL-20 mRNA expression (Figure 6B), p38 MAPK and Akt phosphorylation (Figure 6C), and NF-kB p65 activation (Figure 6D).

4 | Discussion

As a potent proinflammatory cytokine, IL-20 significantly contributes to the pathogenesis of several inflammatory diseases. It induces the production of other inflammatory mediators, which promotes the activation and recruitment of immune cells to sites of inflammation [9, 26]. The elevated level of IL-20 is associated with increased expression of catabolic enzymes, such as matrix metalloproteinases, which degrade the extracellular matrix, leading to tissue destruction and migration of cells [27]. Studies suggest that IL-20 may be involved in regulating inflammatory responses and promoting cell apoptosis [28], thus negatively impacting IVD structure and function. Additionally, IL-20 may modulate the generation of inflammatory responses, accelerating the degradation of IVD tissues and further aggravating the degenerative process [7]. The role of resistin in stimulating IL-20 gene expression in NP cells may lead to the accelerated progression of IDD. However, the mechanism through which resistin regulates IL-20 gene expression in these cells remains unclear. Our findings are significant in

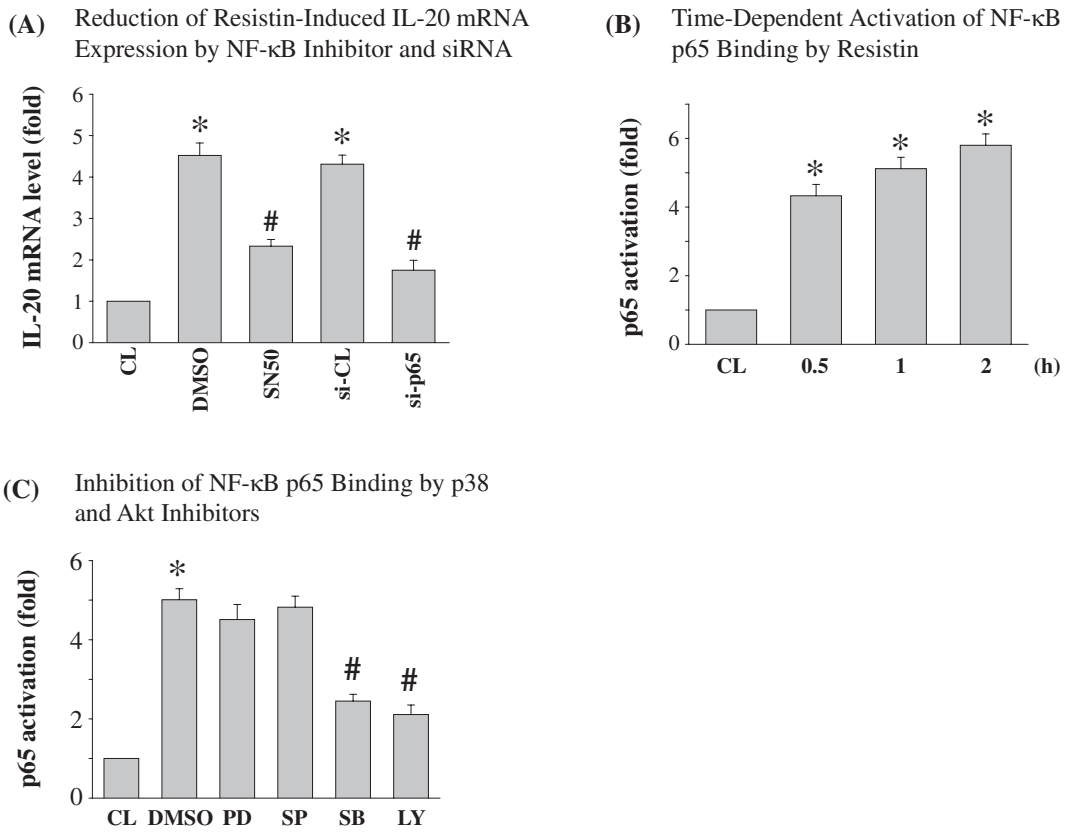

FIGURE 3 | Induction of NF-kB-p65 activity by resistin stimulation in NP cells. (A) IL-20 mRNA expression levels were determined in NP cells pretreated with vehicle (DMSO) or SN50, or transfected with control siRNA (si-CL) or si-p65, and then stimulated with 50 ng/mL resistin for 2 h. RNA samples were isolated and subjected to real-time polymerase chain reaction analysis. Data are presented as fold changes in fluorescent density from control NP cells (CL) normalized to 18S rRNA level. (B, C) The NF-kB p65 activation was determined by a transcription factor (TF)-enzyme-linked immunosorbent assay. (B) NP cells were employed as control (CL) or stimulated with resistin for the durations indicated. (C) NP cells were pretreated individually with PD98059 (PD), SP600125 (SP), SB203580 (SB), or LY294002 (LY) for 1 h before being used as controls (CL) or stimulated with 50 ng/mL resistin for 1 h. NF-κB p65 activity was then analyzed. All bar graphs represent folds of CL NP cells, mean ± standard error of the mean. *$p < 0.05$ versus CL. #$p < 0.05$ versus DMSO or si-CL under resistin stimulation.

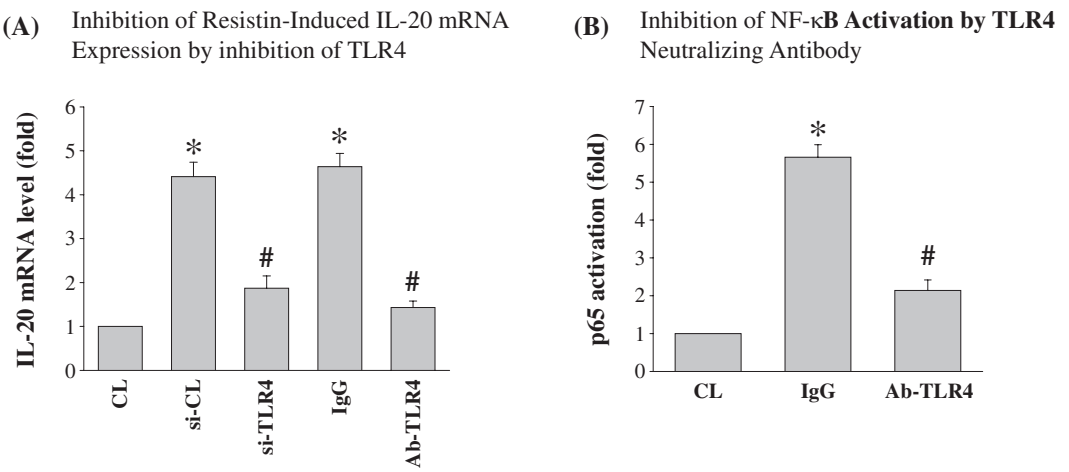

FIGURE 4 | Blockade of TLR4 activity inhibited resistin-induced IL-20 expression. NP cells were kept as controls (CL), or pretreated with isotype-matched IgG (Ab-IgG) and specific TLR4 neutralizing antibody (Ab-TLR4), or transfected with the control siRNA (si-CL) and si-TLR4, and subsequently stimulated with resistin for 2 h (A) and 1 h (B). (A) IL-20 mRNA levels were determined through real-time PCR in NP cells and normalized to 18S rRNA. (B) The activation of NF-kB-p65 in NP cells after resistin stimulation was analyzed using transcription factor (TF) ELISA. All bar graphs represent folds of control NP cells (CL), mean ± standard error of the mean. *$p < 0.05$ versus CL NP cells. #$p < 0.05$ versus IgG-pretreated or si-CL-transfected NP cells under resistin stimulation.

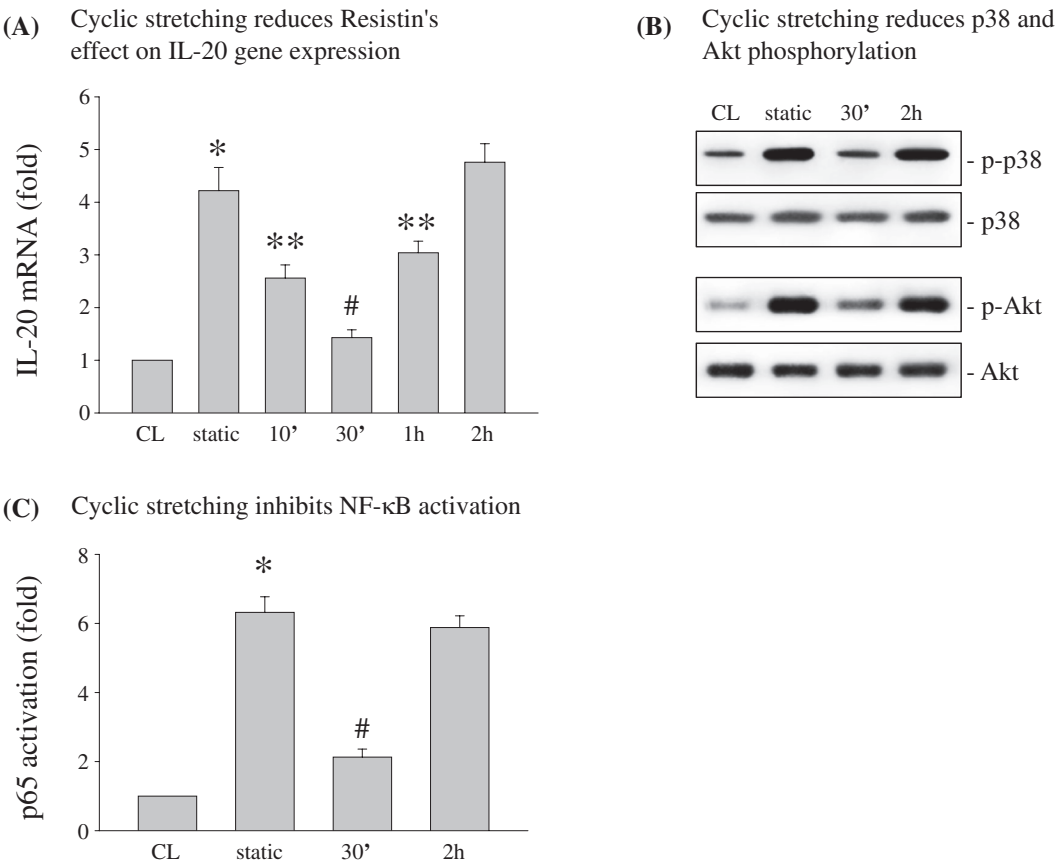

FIGURE 5 | Pre-exposure of NP cells to 5% cyclic stretch with 0.1 Hz for 30 min inhibited resistin-induced IL-20 expression. Static NP cells were stimulated with resistin without prestretching (static). NP cells were kept as controls (CL) or pre-exposed to cyclic stretch at 5% with 0.1 Hz for the indicated durations followed by resistin stimulation. (A) The mRNA levels of IL-20 in NP cells were determined through real-time polymerase chain reaction and normalized to 18S rRNA. *$p < 0.05$ versus CL NP cells. **$p < 0.05$ versus static NP cells with resistin stimulation. #$p < 0.05$ versus resistin-treated NP cells with cyclic stretch at 5% with 0.1 Hz for 10' and 1 h. (B) The phosphorylation of p38 MAPK and Akt was determined by Western blotting. (C) NF-kB-p65 activation in NP cells after 1 h resistin stimulation was analyzed by TF-ELISA. All bar graphs represent folds of control NP cells (CL), mean ± standard error of the mean. *$p < 0.05$ versus CL NP cells. #$p < 0.05$ versus static NP cells with resistin stimulation.

several key ways (Figure 7): (1) resistin stimulates IL-20 mRNA expression and protein secretion in NP cells, (2) TLR4, p38 MAPK, and Akt phosphorylation, and the activation of NF-kB mediate resistin-induced IL-20 expression in NP cells, and (3) low-frequency tensile stretch attenuates resistin-induced IL-20 expression by upregulating NRF2 expression.

Obesity, a risk factor, impacts the health and function of IVD, potentially hastening degenerative processes [12]. According to the study reported by Li et al., the healthy IVD tissues exhibited low levels of resistin, but that were elevated in the patient tissues with severe IDD, suggesting a potential role for resistin in the development of IDD [29]. In our investigation, resistin can potentially induce the development of IDD through inflammation [30, 31]. Resistin upregulates the expression of protease ADAMTS-5 in rat NP cells, a key enzyme involved in extracellular matrix degradation [32]. Resistin has been also found to increase the expression of CCL4, a proinflammatory chemokine, in degenerated human NP tissues through its interaction with TLR4 [29]. Additionally, enhanced levels of IL-20 in individuals with obesity [33] suggest a significant role of IL-20 in IDD development. Consequently, stimulation by resistin might enhance IL-20 secretion and accumulation in IVD tissues, exacerbating the inflammatory response and potentially escalating the IDD progression. This study shows that resistin not only promotes the secretion of IL-20 but also induces its transcription and expression in NP cells. We also observed that TLR4, p38 MAPK, and Akt phosphorylation activate NF-kB in response to resistin. TLR4 has been shown to activate NF-kB, an important transcription factor, in a variety of cell types, including IVD cells [34–36]. Resistin has also been reported to activate proinflammatory pathways through TLR4 receptors and affect various cellular functions [37]. In this study, we utilized TF-ELISA to show that increased NF-kB p65-DNA-binding activity regulated the expression of IL-20 in NP cells. Previous studies have shown the molecular mechanism involved in NF-kB-mediated activation of IL-20 in other cells [22]. Based on our findings, we suggest a signal transduction pathway in NP cells in which resistin functions along with TLR4, and turns on NF-kB to initiate IL-20 transcription. Despite these promising findings, further clinical evidence is needed to fully elucidate the implications of resistin in the complex pathophysiology of IVD.

The IVD tissue is constantly exposed to mechanical forces from daily activities and body weight stress, impacting the regulation of gene expression in NP cells under mechanical forces [38]. The

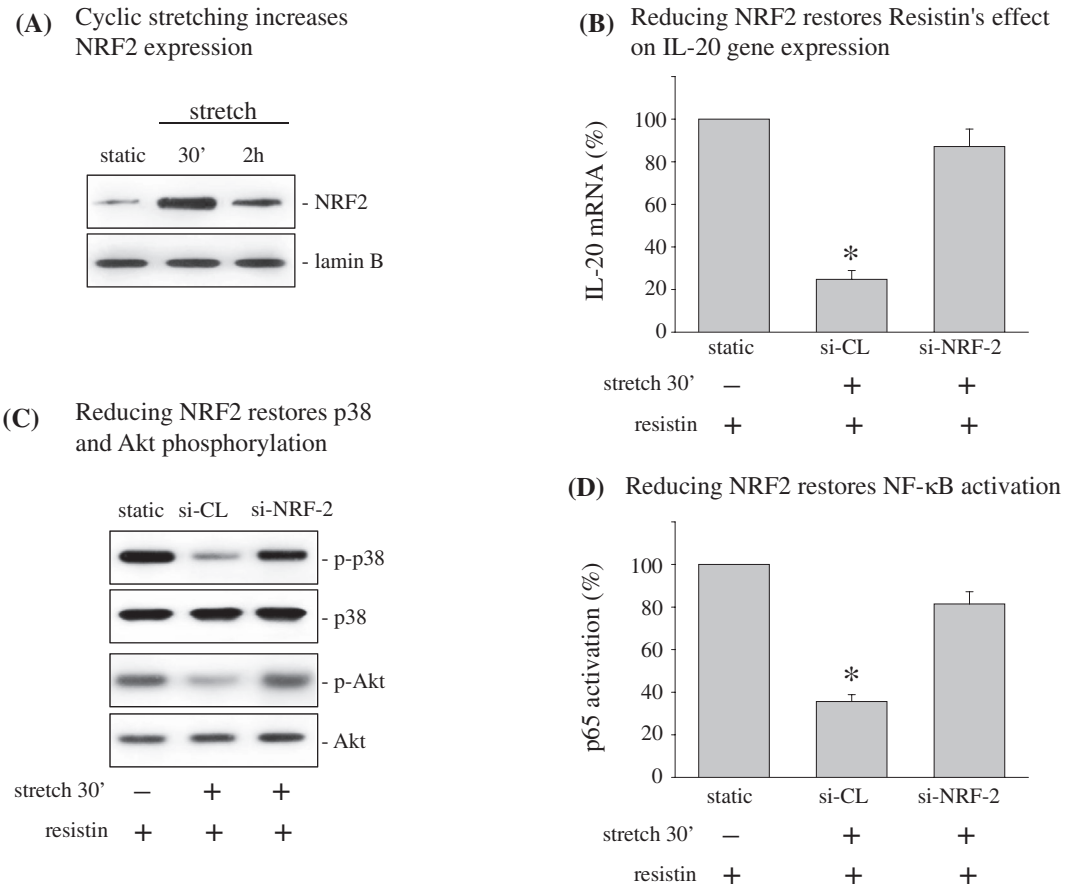

FIGURE 6 | Upregulation of NRF2 inhibited resistin-induced IL-20 expression in NP cells. (A) NP cells were used as static control (static) or exposed to 5% with 0.1 Hz cyclic stretch for 30 min or 2 h. The expression of NRF2 in the nucleus was determined by Western blotting. (B–D) NP cells were used as static control (static), or pre-exposed to 5% with 0.1 Hz for 30 min, and then treated with resistin (50 ng/mL) for 2 h (B), 30 min (C), and 1 h (D). Prior to cyclic stretch exposure, NP cells were transfected with the control siRNA (si-CL) or si-NRF2. (B) The levels of IL-20 mRNA in NP cells were determined through real-time polymerase chain reaction and normalized to 18S rRNA. (C) The phosphorylation of p38 MAPK, and Akt was determined by Western blotting. (D) NF-kB-p65 activation in NP cells was analyzed by TF-ELISA. All bar graphs represent the percentage of static NP cells (static), mean ± standard error of the mean. *$p < 0.05$ versus static NP cells.

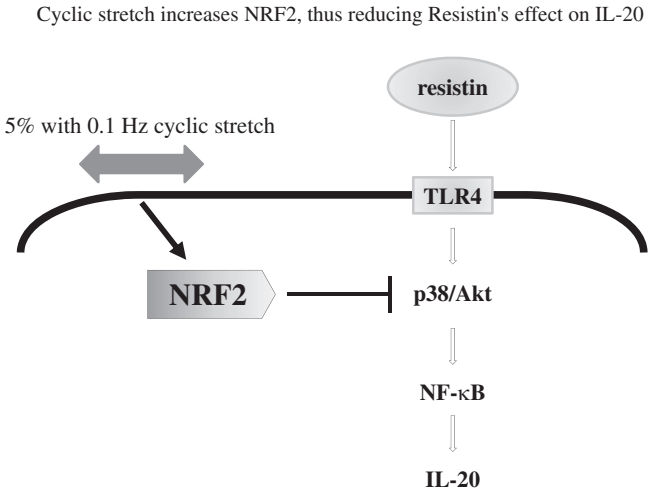

FIGURE 7 | Schematic representation of the signaling pathways regulating 5% with 0.1 Hz cyclic stretch-induced NRF2 expression and consequent inhibition of resistin effect in human NP cells.

magnitude of the force of mechanical stretching crucially influences IVD structure and gene expression [39]. We observed that short-term (30 min) low-frequency stretching induces signaling pathways that regulate gene expression and provides a counterbalance to the progression of inflammatory responses in NP cells; in contrast, long-term (2 h) low-frequency stretching does not yield significant effects. We found that short-term low-frequency stretching prevents resistin from activating NF-kB and IL-20 expression in NP cells, suggesting that stretching NP cells at low frequencies over a short-term duration stops both resistin from activating NF-kB and the expression of IL-20.

Nrf2 and NF-κB significantly interplay within tissues, collaboratively modulating mechanical stress and inflammatory responses in human cells [17]. Nonetheless, their precise roles in IVD tissue remain underexplored. NF-κB, when activated by external stimuli, orchestrates the regulation of inflammatory responses and cellular injury mechanisms, the expression of proinflammatory cytokine genes [40, 41]. In contrast, Nrf2 has a negative regulatory effect on the NF-κB signaling pathway through various intracellular pathways [42]. We observed that Nrf2, induced by short-term low-frequency stretching, serves a protective role in human NP cells and diminishes the influence of resistin on NF-κB activation. This anti-inflammatory function of Nrf2 in human NP cells aligns with its already established role

in other cell types, where it inhibits NF-κB binding to the promoters of inflammatory adhesion molecule genes. Consequently, we propose that Nrf2 induction under short-term low-frequency stretching in human NP cells may act as a mechanomediator, thereby establishing a protective mechanism that mitigates inflammatory responses in IVD (Figure S1).

5 | Conclusion

This study established the link between resistin and IL-20 expression in NP cells and uncovered the molecular pathways encompassing resistin-induced p38 MAPK and Akt phosphorylation, NF-κB activation, and IL-20 expression in these cells. Additionally, this study also validates that short-term, low-frequency stretching-induced NRF2 expression recovers the inflammatory impact of resistin on NP cells. Our findings offer a molecular mechanism involved in the protection conferred by tensile forces against IL-20.

6 | Study Limitations

Although our study design does not fully replicate the typical physiological conditions experienced by IVD in vivo, we believe our findings offer insights into the potential effects of short-duration mechanical stimuli relevant to physiological and pathological conditions. The NP cells in the human IVD are primarily subjected to compressive rather than tensile stress during daily activities, with only occasional exposure to tensile forces. Interestingly, Matsumoto et al. have used cyclic tensile stretch to increase the motion and stress of IVD and found cyclic stress on NP cells can promote the proliferation of cells and alter the properties of IVD cells [38]. Therefore, while our use of cyclic tensile stress provides a model to study certain mechanical responses, it does not entirely simulate the sustained compressive forces that NP cells endure in vivo. Moreover, although our findings highlight the response of NP cells to short-duration mechanical stimuli, more research is needed to explore the long-term effects of mechanical forces that more closely mimic the actual loading patterns within the spine. Future studies should focus on more physiologically relevant models, such as three-dimensional culture systems or in vivo experiments, to validate these results and better understand the effects of long-term mechanical loading on NP cells. In addition, while our study provides valuable insights into the potential effects of resistin and mechanical stimulation on NP cells, it is important to acknowledge that the use of commercially available NP cells cultured in monolayer for multiple passages prior to the experiment may not accurately represent the in vivo environment [43]. Monolayer culture may alter gene expression and cellular behavior over time, potentially impacting the cell's response to stimuli. Therefore, additional studies utilizing more advanced models, such as three-dimensional cultures or organotypic systems, are necessary to confirm these observations in a more realistic biological context.

Author Contributions

Chia-Kung Yen, Hung-Yu Pan, Cheng-Nan Chen, and Kuo-Yuan Huang contributed substantially to the conception and design of the work. Chia-Kung Yen and Hung-Yu Pan performed the acquisition and interpretation of literature data, computational modeling, analysis, presentation and interpretation of results, drafting of the article, revising it critically, and final approval. Hung-Yu Pan, Hsin-I Chang, and Ying-Chen Lu contributed to the acquisition of laboratory data, analysis, and interpretation of results. Cheng-Nan Chen and Kuo-Yuan Huang as the overall project funding holder took responsibility for the integrity of the work from inception to the finalized article and provided substantial contribution to data interpretation and presentation. Yeau-Ren Jeng, Cheng-Nan Chen, and Kuo-Yuan Huang drafted the manuscript. All authors critically revised the manuscript and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.