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Dulaglutide mitigates inflammatory response in fibroblast-like synoviocytes

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ABSTRACT

Rheumatoid arthritis is a common autoimmune disease primarily characterized by chronic inflammation, the formation of an invasive pannus, and destruction of the joints. In the present study, we employed real-time PCR and western blot analysis to investigate the role of dulaglutide in human fibroblast-like synoviocytes (FLS). The results of our study show that dulaglutide exerted a powerful protective effect by rescuing mitochondrial membrane potential, inhibiting the production of NOX-4, and abrogating TNF-α-induced downregulation of the antioxidant GSH. Our findings demonstrate that dulaglutide significantly ameliorated the expression of proinflammatory cytokines and chemokines including IL-1β, IL-6, MCP-1, and HMGB-1. Matrix metalloproteinases mediate cartilage destruction, thereby aiding in pannus formation. Our findings indicate that dulaglutide treatment significantly downregulated the expression of MMP-3 and MMP-13, two crucial degradative enzymes. Importantly, the results of our study demonstrate that the beneficial effects of dulaglutide are mediated through the JNK/NF-κB signaling pathway, which has been suggested as a potential treatment target against RA. Taken together, the results of this study show that dulaglutide may exert significant protective effects against the progression of RA induced by TNF-α.

1. Introduction

One of the main characteristics of rheumatoid arthritis (RA) is the initiation of an autoimmune reaction targeting the joints. Specifically, the synovium undergoes a degradative process that leads to a loss of space between the bones [1]. While the reason for the development of this destructive autoimmune response remains unclear, it is suspected to be the result of both epigenetic and hereditary factors [2]. The symptoms, areas affected, and intensity of RA can vary significantly from patient to patient, and therefore, it can be challenging to diagnose the disease accurately. Fibroblast-like synoviocytes (FLS) are the most abundant cell type comprising the synovial membrane and one of the most significant study targets in RA research. FLS play a critical role in the normal function of the synovium, but during the development of RA, FLS undergo a process of dysregulation induced by cytokines and chemokines, such as interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein (MCP)-1. Overexpression of these factors causes FLS to migrate to surrounding tissue where they infiltrate bone and cartilage, thereby causing sustained inflammation and damaging adjacent tissues. This excessive production of cytokines and chemokines is induced by a combination of factors including mitochondrial dysfunction and oxidative stress [3]. Reduced mitochondrial membrane potential ($_{\Delta}\Psi m$) is a determinate of mitochondrial dysfunction in FLS. Mitochondrial mutagenesis and disrupted mitochondrial membrane potential have been shown to contribute to the inflammatory microenvironment in RA [4]. Oxidative stress is identified by the presence of reactive oxygen species (ROS) in FLS, and in the RA-afflicted synovium, production of ROS is severely increased [5]. Tumor necrosis factor alpha (TNF-α), the primary effector in RA and other chronic inflammatory diseases, is recognized as a causative factor in the pathogenesis of RA. Upon exposure to TNF-α, the longevity and overall health of FLS are significantly reduced [6]. While TNF-α blockers are a commonly used treatment for RA with generally good efficacy, some patients do not respond to such treatment, and therefore, it is necessary to develop alternative therapies against RA [7].

Dulaglutide is a specific agonist of the receptor for the peptide hormone glucagon-like peptide-1 (GLP-1R). Agonism of GLP-1R has been shown to produce anti-inflammatory and other beneficial effects that may ameliorate the pathological processes of RA. Dulaglutide has been shown to exert numerous beneficial effects in the treatment of diseases including type 2 diabetes [8]. In a 2015 study, dulaglutide was found to reduce glycated hemoglobin in patients with type II diabetes [9]. Agonism of GLP-1 has also been shown to increase bone formation and metabolism and to effectively counter bone loss in previously obese women [10]. In the present study, we investigated the effects of GLP-1 agonism by dulaglutide in the context of RA by performing a series of in

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vitro experiments. Our findings demonstrate that dulaglutide treatment may be a safe and beneficial therapy against chronic inflammation, invasive pannus formation, and oxidative stress in RA. Importantly, we demonstrate that the effects of dulaglutide are mediated through the C-Jun N-terminal kinase (JNK)/NF-κB signaling pathway.

2. Materials and methods

2.1. FLS isolation and treatment

Experimental protocols with human subjects were designed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Human subject experiments were approved by the ethics committee of our institute. Written informed consent was signed with all participants. FLS were isolated from knee joint synovial tissues from 15 donors. Samples were collected and minced into small pieces. Samples were digested with 0.05% trypsin for 10 min at 37 °C. Cells were collected and seeded at a density of 1.5×10^6 cells per well in 35 mm diameter cell culture dishes. FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h.

2.2. Real-time polymerase chain reaction (PCR) analysis

After appropriate stimulation, total intracellular RNA was isolated from FLS using Qiazol reagent (Qiagen, USA). A NanoDrop ND1000 spectrophotometer was used to determine the concentration and quality of isolated RNA. Isolated RNA (1 μg) was used to produce cDNA via reverse transcription PCR (RT-PCR) using the iScript RT-PCR kit (Bio-Rad, USA). Expressions of target genes were measured using a real-time PCR analysis on a 7500 Real-Time PCR System (Applied Biosystems, USA) using a commercial SYBR Green PCR Master Mix kit (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used in this study:

NOX-4 (For, 5′-CTTTTGGAAGTCCATTTGAG-3′; Rev, 5′-CGGGAGG GTGGGTATCTAA-3′); IL-6 (For, 5′-GGTACATCCTCGACGGCA TCT-3′; Rev, 5′- GTGCCTCTTTGCTGCTTTCAC-3′); MCP-1 (For, 5′-ATGCAATCAATGCCCCAGTC-3′; Rev, 5′-TGCAGATTCTTGGGTT GTGG-3′); IL-1β (For, 5′- TTCCTGTTGTCTACACCAATGC-3′; Rev, 5′-CGGGCTTTAAGTGAGTAGGAGA-3′);
MMP-3 (For, 5′-CCTCTATGGACCTCCCACAGAATC-3′; Rev, 5′-GGT GCTGACTGCATCGAAGGACAAA-3′); MMP-13 (For, 5′- CTGGCCTG CTGGCTCATGCTT-3′; Rev, 5′-CCTCAGAAAGAGCAGCATCGAT ATG-3′); GAPDH (For, 5′-ACT GGCGTCTTCACCACCAT-3′; Rev, 5′-AAG GCC ATG CCA GTG AGC TT-3′).

2.3. Western blot analysis

After appropriate stimulation, FLS were lysed with cell lysis buffer (Cell Signaling Technologies, USA) containing the phosphatase and protease inhibitor cocktail. Then, a 20 μg sample from each group was loaded and separated for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). Samples were blocked with 5% skim milk for 2 h at room temperature (RT). Membranes were then incubated with primary antibodies overnight at 4 °C. After 3 washes with PBS, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at RT. Immuno-bands were visualized with an enhanced chemiluminescence (ECL) kit (GE Healthcare, USA). The following antibodies were used in this study: NOX-4 (#ab133303, Abcam, USA); NF-kB p65 (#ab32536, Abcam, USA); Lamin B (#ab133741, Abcam, USA); JNK (#ab129377, Abcam, USA); phospho-JNK (#ab46821, Abcam, USA); IκBα (#ab32518, Abcam, USA); p-IκBα (#ab92700, Abcam, USA); β-actin (1:10000, #3700, Cell Signaling Technology, USA); Aggrecan (1:1000, #ab3778, Abcam, USA); Anti-rabbit IgG, HRP-linked secondary antibody (1:3000, #7074, Cell Signaling Technology, USA); Anti-mouse IgG, HRP-linked antibody (1:3000, #7076, Cell Signaling Technology, USA).

2.4. Dihydroethidium (DHE) staining

Superoxide production in FLS was assessed by staining cells with the fluorescent dye DHE. FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. Cells were stained with 2 μM DHE (Thermo Fisher Scientific, USA) in phenol-free red medium at 37 °C for 30 min. After 3 washes, fluorescent signals were visualized and recorded with a fluorescence microscope (Nikon, Japan). The fluorescence density of DHE in FLS was calculated using Image J software (NIH, USA). We defined regions of interest (ROI) and counted the number of cells in the ROI. We then assessed the integrated density value (IDV) of the ROI. The level of ROS = IDV/cell number.

2.5. Determination of reduced glutathione (GSH)

Intracellular levels of reduced glutathione (GSH) in FLS were measured using a fluorometric assay. After the necessary treatment, FLS were collected and centrifuged at 14000 × *g* for 5 min. Supernatants were collected and mixed with OPAME in methanol and borate buffer. After incubation for 15 min at RT, fluorescent signals were recorded at the wavelength at 350 nm excitation and 420 nm emission.

2.6. Measurement of mitochondrial membrane potential ($_{\Delta}\Psi\boldsymbol{m}$)

Intracellular levels of mitochondrial membrane potential ($_{\Delta}\Psi\boldsymbol{m}$) in FLS were assessed using tetramethylrhodamine methyl ester (TMRM). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. Cells were then incubated with 20 nmol/L TMRM for 1 h at RT. Cells were washed 3 times, and fluorescence signals were detected using an IBE2000 inverted fluorescence microscope (Zeiss, Germany). The fluorescence density of TMRM in FLS was calculated using Image J software (NIH, USA). We defined regions of interest (ROI) and counted the number of cells in the ROI. We then assessed the integrated density value (IDV) of the ROI. The level of $_{\Delta}\Psi\boldsymbol{m}$ = IDV/cell number.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Secreted levels of IL-1β, IL-6, MCP-1, high-mobility group box 1 (HMGB-1) and expressions of MMP-3 and MMP-13 in FLS were measured using commercial ELISA kits: IL-1β (#DLB50, R&D Systems), IL-6 (#D6050, R&D Systems), MCP-1 (#DCP00, R&D Systems), HMGB-1 (#ABIN414391, Cloud-Clone Corp), MMP-3 (#DMP300, R&D Systems), and MMP-13 (#DY511, R&D Systems). Plates were coated with primary antibodies against target proteins overnight at 4 °C. Plates were then washed 3 times with PBS and blocked with goat serum at RT for 1 h. Next, 50 μl samples were added to each well of the ELISA plates and incubated at 4 °C overnight. Wells were then washed and incubated with biotinylated sheep polyclonal antibodies for 1 h at RT, followed by incubation with 50 μl of avidin-HRP (diluted 1:5000). Reactions were stopped with H_2SO_4. Absorbance recorded at 490 nm was measured to reflect protein concentrations.

2.8. Luciferase reporter gene assay

The transcriptional activity of the nuclear factor-κB (NF-κB) transcriptional factor was measured using a NF-κB promoter-luciferase activity assay. NF-κB promoter-luciferase and β-galactosidase plasmids were purchased from Clontech and transfected into cells with

Lipofectamine 3000 (Thermo Fisher Scientific, USA). At 12 h post transfection, cells were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. Luciferase and β-galactosidase activities were assessed using a dual luminescence assay kit. Luciferase activity was normalized to β-galactosidase activity.

2.9. Statistical analysis

Each experiment was repeated at least 3 times. Experimental data are presented as means ± standard error of measurement (S.E.M.). Statistical analysis was performed using analysis of variance (ANOVA) where appropriate, followed by the Bonferroni post-hoc test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Dulaglutide ameliorates TNF-α-induced mitochondrial dysfunction in human FLS

Human FLS are a crucial component in the pathogenesis of rheumatoid arthritis [11]. Due to their role in the synovium and their proliferation during the development of RA, understanding the effectors involved is crucial to the study and treatment of this disease. In the present study, dulaglutide was found to have a positive effect on alleviating mitochondrial dysfunction in human FLS. We began testing the theorized abilities of dulaglutide by incubating human FLS with 10 ng/mL TNF-α in both the presence and absence of dulaglutide (50, 100 nM) for 24 h. We then tested the levels of mitochondrial membrane potential via TMRM staining. As shown in Fig. 1, exposure of human FLS to TNF-α significantly reduced cellular mitochondrial membrane potential. However, mitochondrial membrane potential was significantly restored to up to 70% of the baseline value by treatment with dulaglutide in a dose-dependent manner.

3.2. Dulaglutide attenuates TNF-α-induced oxidative stress in human FLS

Our next object of study was the effect of dulaglutide on oxidative stress caused by TNF-α. Briefly, FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. We then assessed the generation of the oxidative stress marker ROS via dihydroethidium (DHE) staining as shown in Fig. 2A and measured the levels of the antioxidant reduced glutathione (GSH) as shown in Fig. 2B. We found that when FLS were incubated with TNF-α alone, the intensity of DHE increased approximately 3-fold. However, when incubated with dulaglutide, specifically at the higher dose of 100 nM, the ROS level was restored to near basal levels. Furthermore, the reduced GSH level fell significantly when exposed to TNF-α alone but was also restored to near basal levels upon the addition of dulaglutide.

3.3. Dulaglutide reduces TNF-α-induced expression of NADPH oxidase 4 (NOX-4) in human FLS

We continued by assessing the impact of dulaglutide on NADPH oxidase 4 (NOX-4) levels. FLS incubated with 10 ng/mL TNF-α for 24 h in the presence or absence of dulaglutide (50, 100 nM). We then determined the expression of NOX-4 at the mRNA level via real-time PCR analysis as shown in Fig. 3A, and at the protein level via western blot analysis as shown in Fig. 3B. Past studies have also investigated the effects and abilities of NADPH oxidase in RA [12]. Concordantly, our findings show that NOX-4 mRNA expression was increased roughly 4-fold upon exposure to 10 ng/mL TNF-α alone and was maintained at the lower levels of roughly 2-fold and 1.5-fold above baseline in the presence of 50 nM and 100 nM dulaglutide, respectively. Additionally, NOX-4 protein expression was increased to roughly 3-fold baseline upon exposure to 10 ng/mL TNF-α alone, while the addition of 50 nM and 100 nM dulaglutide resulted in increases of only roughly 2-fold and 1.5-fold, respectively.

3.4. Dulaglutide suppresses TNF-α-induced expression and secretion of pro-inflammatory cytokines in human FLS

Next, we investigated the effects of dulaglutide on TNF-α-induced cytokine expression. Briefly, FLS were incubated with 10 ng/mL TNF-α for 24 h in the presence or absence of dulaglutide (50, 100 nM). We

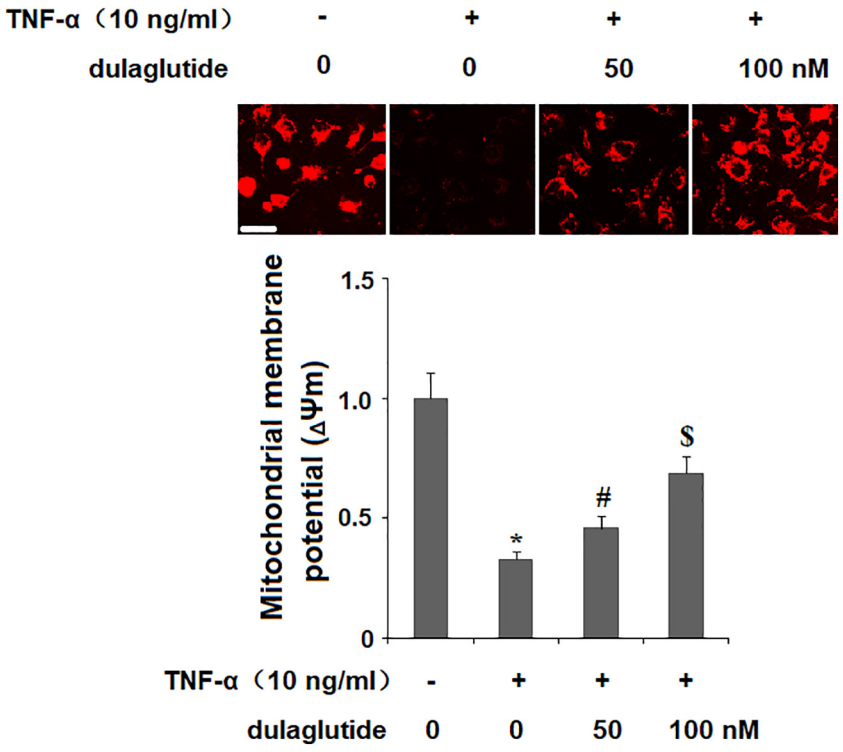

Fig. 1. Dulaglutide ameliorated TNF-α-induced mitochondrial dysfunction in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. Levels of mitochondrial membrane potential ($_{\Delta}\Psi$m) was determined by TMRM staining, Scale bars, 100 μM (*, #, $, $P < 0.01$ vs. previous column group, $n = 5$–6).

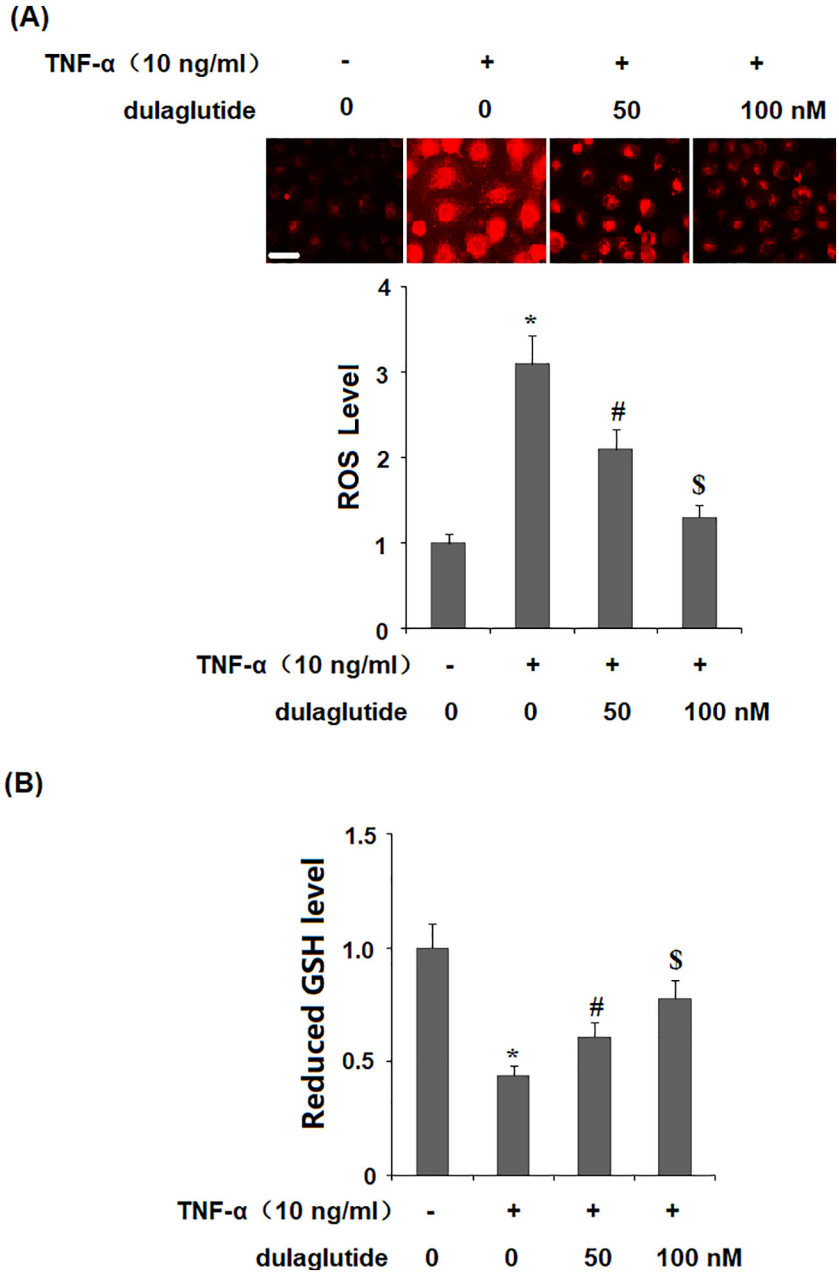

Fig. 2. Dulaglutide attenuates TNF-α-induced oxidative stress in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. (A). The generation of the oxidative stress marker dihydroethidium (DHE) was assessed by staining; (B). The levels of antioxidant reduced glutathione (GSH) were measured, Scale bars, 100 μM (*, #, $, $P < 0.01$ vs. previous column group, $n = 5–6$).

measured the expression of IL-1β, IL-6, and MCP-1 at both the mRNA and protein levels via real-time PCR and ELISA analyses, respectively. Upon exposure to 10 ng/mL TNF-α alone, the mRNA expression of IL-1β, IL-6, and MCP-1 increased approximately 3-fold, 4-fold, and 3.5-fold, respectively. However, when also exposed to dulaglutide, and most notably the higher dose of 100 nM, expression of IL-1β and MCP-1 could be maintained near basal levels, while IL-6 increased only roughly 2-fold. At the protein level, when exposed to 10 ng/mL TNF-α alone, the expressions of IL-1β, IL-6, and MCP-1 increased by roughly 3-fold, while dulaglutide decreased the expression of these cytokines to near baseline, with the higher dose of 100 nM dulaglutide being more effective. Next, we tested the ability of dulaglutide to suppress TNF-α-induced secretion of HMGB-1 in FLS. We found that upon exposure to 10 ng/mL TNF-α alone, the expression of HMGB-1 increased roughly 2.5-fold. However, treatment with dulaglutide reduced the expression of HMGB-1 to near basal levels in a dose-dependent manner. Finally, we tested the effect of dulaglutide on the expression of MMP-3 and MMP-13. Previous studies have linked these cytokines to the development of arthritis in mouse models [13]. To test this, we incubated FLS with 10 ng/mL TNF-α for 24 h in the presence or absence of dulaglutide (50, 100 nM) and proceeded to test the mRNA and protein expression levels of MMP-3 and MMP-13 via real-time PCR and ELISA, respectively. We found that at the mRNA level, the expressions of MMP-3 and MMP-13 increased roughly 4-fold upon exposure to 10 ng/mL TNF-α alone. However, the addition of dulaglutide reduced the increase in expression of these enzymes to only roughly 2-fold. At the protein level, the expressions of MMP-3 and MMP-13 increased roughly 3-fold upon exposure to TNF-α alone, but the addition of 50 nM and 100 nM dulaglutide reduced the protein expression of these enzymes to only roughly 2-fold baseline in a dose-dependent manner.

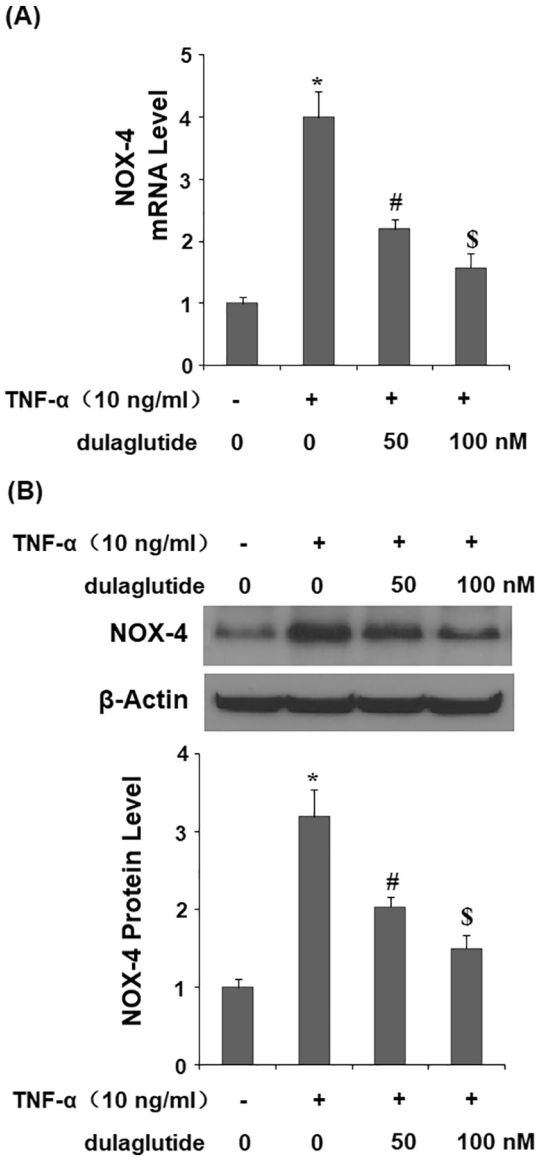

Fig. 3. Dulaglutide reduces TNF-α-induced expressions of NADPH oxidase 4 (NOX-4) in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. (A). Expression of NOX-4 at the mRNA levels was determined by real-time PCR analysis; (B). Expression of NOX-4 at the protein levels was determined by western blot analysis (*, #, $, $P < 0.01$ vs. previous column group, $n = 5–6$).

3.5. Dulaglutide prevents TNF-α-induced activation of JNK in human FLS

We then went on to investigate the effect of dulaglutide on the activation of the proinflammatory c-Jun N-terminal kinase (JNK) signaling pathway, which is a popular treatment target in RA. Similarly, another GLP-1 agonist, exendin-4, was shown to regulate the activation of the JNK pathway in past studies. Exendin-4 prevented vascular smooth muscle cell proliferation and migration induced by angiotensin II via inhibition of the ERK1/2 and JNK signaling pathways [14]. In the current study, we incubated FLS with 10 ng/mL TNF-α for 2 h in the presence or absence of dulaglutide (50, 100 nM) and proceeded to measure the phosphorylated and total levels of JNK via western blot analysis. β-Actin was used as a control in this experiment. Our findings indicate that exposure to TNF-α significantly increased the level of phosphorylated JNK, while the level of total JNK remained constant. However, with the addition of dulaglutide, the level of phosphorylated JNK was maintained at basal levels due to the prevention of TNF-α-induced phosphorylation. This indicates that agonism of GLP-1R by dulaglutide can inhibit the activation of the JNK pathway. Interestingly, GLP-1 has also been shown to reduce macrophage infiltration and inflammation in a mouse model [15].

3.6. Dulaglutide prevents TNF-α-induced phosphorylation and degradation of IκBα and activation of NF-κB in human FLS

Finally, we examined the effects of dulaglutide on the phosphorylation of IκBα and subsequent activation of NF-κB in FLS. NF-κB is well-recognized as playing a significant role in the pathogenesis of RA and other inflammatory diseases and is considered a valuable treatment target [16]. In the present study, we incubated FLS with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 6 h when studying Iκ-Bα and for 24 h when studying NF-κB. Using western blot analysis for the study of Iκ-Bα, we found that TNF-α alone increased the level of phosphorylated IκBα by nearly 3-fold, but the addition of dulaglutide mitigated this increase to around 1.5-fold. Using western blot analysis for the study of NF-κB, we found that when exposed to TNF-α alone, p65 levels increased by roughly 3-fold and NF-κB luciferase activity increased nearly 20-fold. However, these effects were greatly reduced upon treatment with dulaglutide. Levels of nuclear p65 levels returned to near-baseline, and NF-κB luciferase activity was reduced to a significantly lower 5-fold increase.

4. Discussion

There are many critical aspects involved in the pathogenic processes of RA, including oxidative stress, chronic inflammation, and destruction of cartilage and bone. Novel therapies that can mitigate these destructive events through modulation of specific signaling pathways have been receiving increasing attention. Mitochondrial membrane potential is significant in the process of energy storage and oxidative phosphorylation and is severely disrupted in RA, thereby leading to an oxidative stress environment [17]. In the present study, we found that treatment with the commonly used anti-diabetes agent dulaglutide significantly rescued reduced mitochondrial membrane potential and the imbalance in the ratio of oxidants to antioxidants induced by exposure to TNF-α, thereby indicating a novel anti-oxidative stress capacity of GLP-1R agonism via rescue of mitochondrial dysfunction. Additionally, dulaglutide increased the level of the antioxidant GSH (Figs. 1–3). Overexpression of proinflammatory cytokines plays a significant role in the progression of RA. Currently, therapies targeting IL-6 are commonly used for the treatment of RA, but the exact mechanisms driving overproduction of IL-6 and other cytokines remain poorly understood [18]. Here, we found that dulaglutide treatment could significantly prevent overexpression of IL-6 and IL-1β at both the mRNA and protein levels. Recruitment of macrophages to the synovium plays a significant role in invasive pannus formation and chronic inflammation. MCP-1 drives the recruitment of immune cells into the synovium, and increased levels of MCP-1 are considered an early warning sign of RA [19]. Here, we found that dulaglutide could effectively diminish the expression of MCP-1, thereby suppressing synovial macrophage infiltration. Additionally, dulaglutide inhibited secretion of HMGB-1 which plays a vital role in RA by facilitating immune and inflammatory response activation and mediating joint tissue homeostasis [20]. Our results show that GLP-1R agonism inhibited the expression of HGMB-1, thereby further regulating inflammation in FLS.

Expression of MMP-3 and MMP-13 is significantly upregulated in RA and these enzymes degrade the components of the articular extracellular matrix, such as type II collagen. Inhibition of MMP-3 and MMP-13 expression has been cited as a potential treatment strategy in RA [21,22]. Our findings demonstrate that dulaglutide significantly decreases expression of MMP-3 and MMP-13 induced by TNF-α in human FLS, thereby suggesting a strong ability of GLP-1R to mediate cartilage homeostasis. Finally, we show that the effects of dulaglutide are

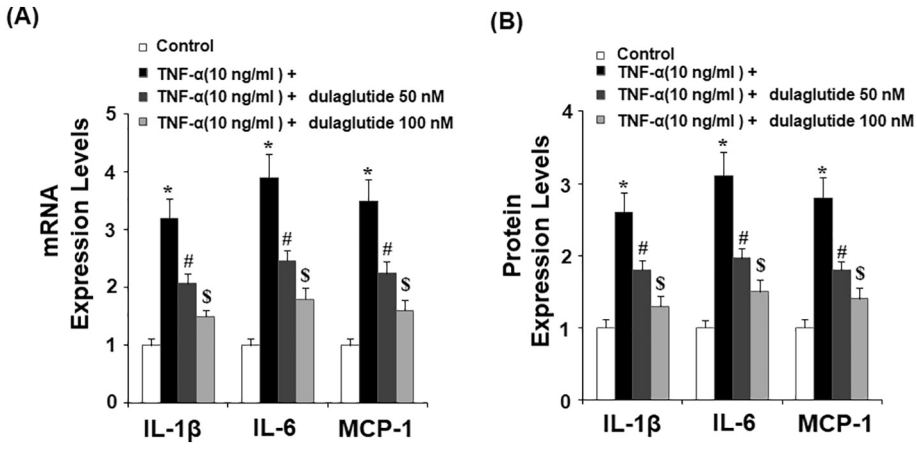

Fig. 4. Dulaglutide suppresses TNF-α-induced expression and secretion of pro-inflammatory cytokines in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. (A). Expression of IL-1β, IL-6, and MCP-1 at the mRNA levels was determined by real-time PCR analysis; (B). Expression of IL-1β, IL-6, and MCP-1 at the protein levels was determined by ELISA (*, #, $, $P < 0.01$ vs. previous column group, $n = 5–6$).

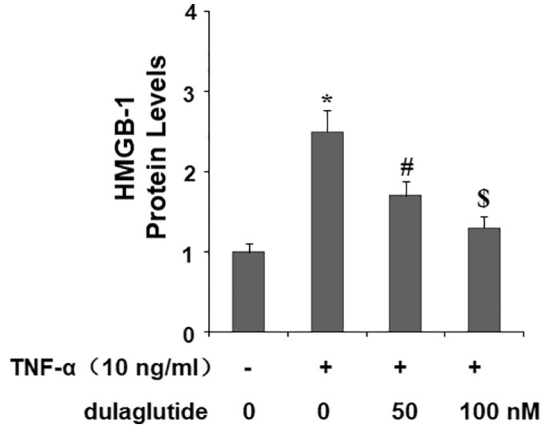

Fig. 5. Dulaglutide inhibits TNF-α-induced the secretions of high-mobility group protein 1 (HMGB-1) in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. Secretions of HMGB-1 were determined by ELISA (*, #, $, P < 0.01 vs. previous column group, n = 5–6).

mediated via the JNK/NF-κB signaling pathway. Inactivation of the JNK pathway is well-recognized as a promising treatment approach for RA. Inhibition of JNK phosphorylation has been shown to suppress migration of RA-FLS, thereby playing a preventative role in pannus formation [23]. Here, we found that dulaglutide could potentially slow FLS migration by downregulating expression of the chemokine MCP-1 as well as the level of phosphorylated JNK. Under normal conditions, the nuclear translocation of p65 protein and resulting activation of the NF-κB signaling pathway is inhibited by IκBα. However, in RA, phosphorylation of IκBα negates its inhibitory effect, allowing p65 to translocate to the nucleus where it activates the NF-κB pathway [24,25]. NF-κB has been shown to induce expression of IL-6 and other inflammatory cytokines, and modulation of the NF-κB pathway is considered a valuable treatment approach in numerous chronic inflammatory diseases including RA [26]. In the present study, we found that treatment with dulaglutide prevented phosphorylation of IκBα, which was reflected by reduced NF-κB luciferase activity. This indicates that the anti-RA effects of agonism of GLP-1R by dulaglutide are mediated through the JNK/NF-κB signaling pathway. Taken together, our findings demonstrate the potential of dulaglutide treatment as a novel therapy against RA. Agonism of GLP-1R by dulaglutide significantly ameliorated TNF-α-induced mitochondrial dysfunction and oxidative stress in human FLS, as evidenced by increased mitochondrial membrane potential, decreased production of NOX-4, and increased levels of GSH. Dulaglutide also significantly reduced the expression of IL-1β, IL-6, MCP-1, and HMGB-1, thereby indicating a potent anti-inflammatory and anti-infiltration capacity of dulaglutide. Additionally, dulaglutide downregulated the expression of MMP-3 and MMP-13, two degradative enzymes that play a pivotal role in cartilage destruction and pannus formation. Importantly, we show that these beneficial effects are mediated through the JNK/NF-κB signaling pathway (Figs. 4–9).

GLP-1 is reported to exert diverse anti-inflammatory actions in different cells and tissues [27]. Previous studies have proven that GLP-1 could potentially be used for the treatment of several chronic inflammatory diseases including atherosclerosis, asthma, and psoriasis [28,29]. GLP-1 and its analogs exert their biological functions by interacting with GLP-1R. Several studies have shown that activation of GLP-1R by GLP-1 and its analogs could downregulate NF-κB phosphorylation and nuclear translocation, which governs the expression of

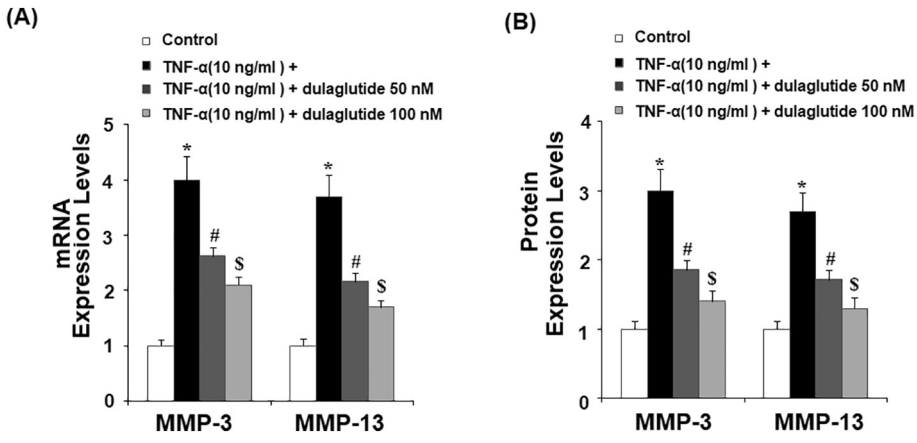

Fig. 6. Dulaglutide inhibits TNF-α-induced the expressions of MMP-3, and MMP-13 in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. (A). mRNA expressions of MMP-3 and MMP-13 were measured by real-time PCR; (B). Protein expressions of MMP- and MMP-13 were measured by ELISA (*, #, $, P < 0.01 vs. previous column group, n = 5–6).

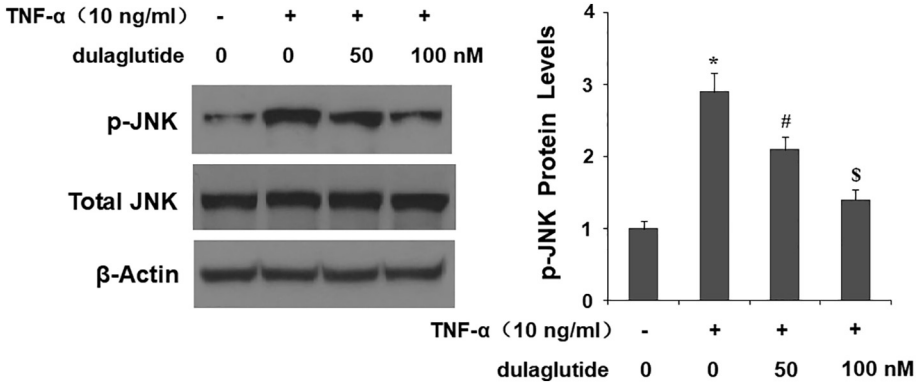

Fig. 7. Dulaglutide prevents TNF-α-induced activation of JNK in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 2 h. Phosphorylated and total levels of JNK were determined by western blot analysis (*, #, $, P < 0.01 vs. previous column group, n = 5–6).

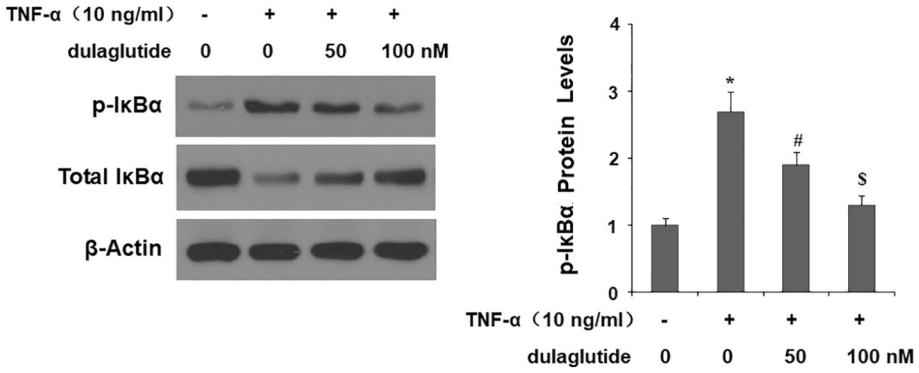

Fig. 8. Dulaglutide prevents TNF-α-induced phosphorylation and degradation of IκBα in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 6 h. Phosphorylated and total levels of IκBα was measured by western blot analysis (*, #, $, P < 0.01 vs. previous column group, n = 5–6).

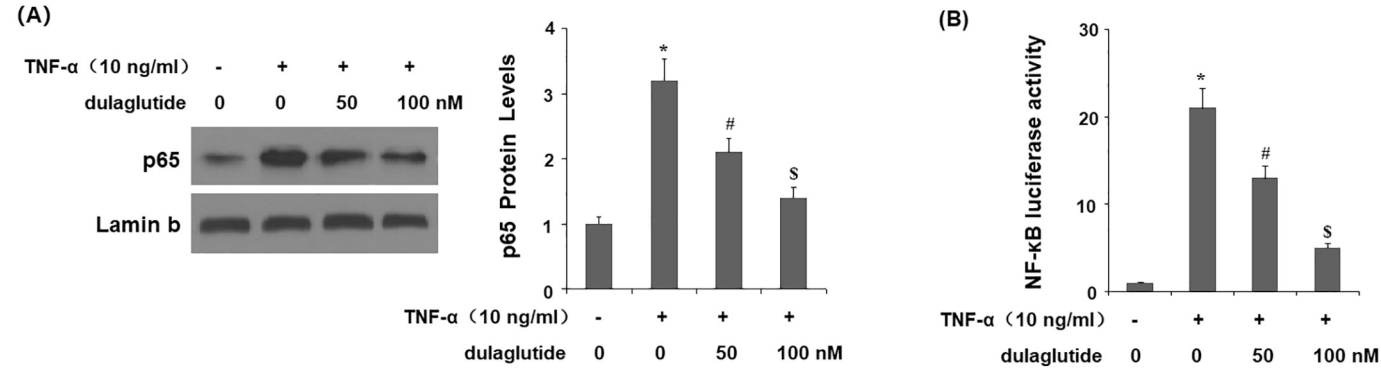

Fig. 9. Dulaglutide prevents TNF-α-induced activation of NF-κB in human fibroblast-like synoviocytes (FLS). FLS were incubated with 10 ng/mL TNF-α in the presence or absence of dulaglutide (50, 100 nM) for 24 h. (A). Nuclear levels of p65 were determined by western blot analysis; (B). Promoter luciferase activity of NF-κB was assayed (*, #, $, P < 0.01 vs. previous column group, n = 5–6).

cytokines, chemokines, and MMPs in a variety of cells and organs. Further research is required to better understand the exact involvement of GLP-1R in the development and progression of RA.

Declaration of Competing Interest

None.

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