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ORIGINAL ARTICLE

Compound GDC, an Isocoumarin Glycoside, Protects against LPS-Induced Inflammation and Potential Mechanisms *In Vitro*

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Abstract— Compound 3*R*-(4′-hydroxyl-3′-O-β-D-glucopyranosyl phenyl)-dihydro isocoumarin (GDC) is a natural isocoumarin, recently isolated from the stems of *H. paniculiflorum.* However, we know little about the effects of GDC on rheumatoid arthritis (RA). This study aims to investigate the protective effects and potential mechanisms of GDC against LPS-induced inflammation *in vitro*. Fibroblast-like synoviocytes (FLSs) obtained from synovial tissue of rats were induced by lipopolysaccharide (LPS) and treated with GDC. Cell viability was determined by mitochondrial-respiration-dependent3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Secretion of various inflammatory mediators was analyzed by ELISA and RayBio® Rat Cytokine Antibody Array. Potential mechanisms that are associated with anti-inflammatory effect were examined by Western blot. Results showed that GDC significantly inhibited the production of tumor necrosis factor alpha (TNF-α) and interleukin- (IL-) 6 induced by LPS. GDC also reduced the expression of inducible nitric oxide synthase (iNOS), TNF-α, IL-6, and IL-1β, as well as proinflammatory cytokines such as activin A, ciliary neurotrophic factor (CNTF), fractalkine, IFN-γ, IL-4, and TIMP-1. Moreover, GDC inhibited LPS-induced phosphorylation of extracellular regulated protein kinases (ERK1/2), p38 mitogen-activated protein kinases (p38), c-Jun N-terminal kinase (JNK), and IκB. And GDC also blocked NF-κBp65 nuclear translocation. All the results suggested that the protective effects of GDC against LPS-induced inflammation *in vitro* may be related with NF-κB and JNK signaling pathway.

KEY WORDS: rheumatoid arthritis; isocoumarin; fibroblast-like synoviocyte; inflammation; NF-κB signaling pathway; MAPK signaling pathway.

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease associated with persistent synovitis and joint damage [1]. Fibroblast-like synoviocytes (FLSs) are considered as an important role in RA development, which increase to 10–15 cell layers due to the influx and proliferation of inflammatory cells when RA occur, and eventually FLSs manifest as pannus formation, which grows in a tumor-like fashion [2–4]. Increased inflammatory immune cells and FLSs produce various cytokines and chemokines, such as tumor necrosis factor alpha (TNF-α), interleukin- (IL-) 6, IL-1β, cyclooxygenase (COX-2), and IL-18, which have been reported to hold the vital role in the pathogenesis of RA, that could in turn promote inflammation in the affected joint [5–7]. So, overcoming the inflammation is still critical for RA therapy.

Recently, there are mainly nonsteroidal anti-inflammatory drugs, corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs), and biological agents such as anti-TNF-α and anti-IL-6-receptor antibodies prescribed to treat RA in clinical. However, these drugs have adverse side effects more or less, such as bone loss, liver failure, respiratory failure, or dermatological damage [8, 9]. On the comparison with the aforementioned conventional Western drugs, natural products and traditional Chinese medicine attract hot attention. 3*R*-(4′-hydroxyl-3′-O-β-D-glucopyranosyl phenyl)-dihydro isocoumarin (GDC) is a natural isocoumarin (Fig. 1), firstly recently isolated from the stems of *H. paniculiflorum* by our group [10]. The genus *Homalium* (Flacourtiaceae) showed a wide variety of interesting bioactivities including anti-bacterial, anti-oxidant, anti-viral, anti-plasmodial, hypolipidemic, and hypoglycemic activities [11–20]. Moreover, recently, it has also been reported that GDC inhibited LPS-induced inflammatory mediators in RAW264.7 cells. However, limited knowledge has been obtained as to how GDC influences the inflammation *in vitro* FLSs; its regulation signals have not been well elucidated.

To address this issue, both the protection of GDC in FLSs following LPS-induced inflammation and its association with anti-inflammatory response signaling pathway were investigated.

MATERIALS AND METHODS

Materials

GDC (purity > 98%, Fig. 1a) was isolated from the stems of *Homalium paniculiflorum* by our group. GDC was dissolved in DMSO, and the final concentration of DMSO was < 0.1% (*v*/*v*). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), collagen type II, lipopolysaccharide (LPS), and DMSO were bought from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), trypsin, and RPMI 1640 were purchased from Hyclone (Logan, UT, USA). Antibodies (IKKβ, p-IκBα, IκBα, NF-κBp65, ERK1/2, p-ERK1/2, p-p38, p38, and β-actin) were purchased from Cell Signaling Technology (Boston, MA, USA). Primary antibodies against p-JNK and JNK were purchased from Abcam (Cambridge, UK). The secondary antibodies conjugated with horseradish peroxidase (HRP) were all bought from Xiamen LuLong Biotech Co., Ltd. (Xiamen, China). All other chemicals and reagents were of analytical grade.

Cell Culture and Treatment

FLSs were isolated and cultured from synovium tissue of Wistar rats as previous studies [14]. In brief, synovium tissues were isolated and digested enzymatically by 0.2% collagen type II (Sigma) in RPMI 1640 for 2 h at 37 °C. The obtained cell suspension was plated onto culture dishes, and cells were used for subsequent experiments between passages 3 and 6.

For all experiments, the cells were seeded on 96-well plates or 6-well plates and were divided into four groups randomly. And cells were treated with either RPMI 1640 with 1% FBS, LPS (1 μg/mL), GDC (3, 30 μM), or LPS (1 μg/mL) + GDC (3, 30 μM), with six wells in each group.

Cell Viability Assay

FLSs were cultured in RPMI 1640 containing concentrations of GDC (0, 3, and 30 μM) with or without 1 μg/mL of LPS for 24 h, and the cell viability was assessed by MTS assay. MTS was added and incubated for an additional 4 h at 37 °C. Then, the absorbance at 570 nm was taken by a microplate reader (Infinite M200 Pro, TECAN).

ELISA for IL-6 and TNF-α

After treatment, culture supernatants were collected and then centrifuged prior to the determination of IL-6 and TNF-α level with a commercially available ELISA kit (ABclonal (Wuhan) biotechnology co. LTD, Wuhan, China). Detailed manipulation process was performed according to the protocol of manufacturer.

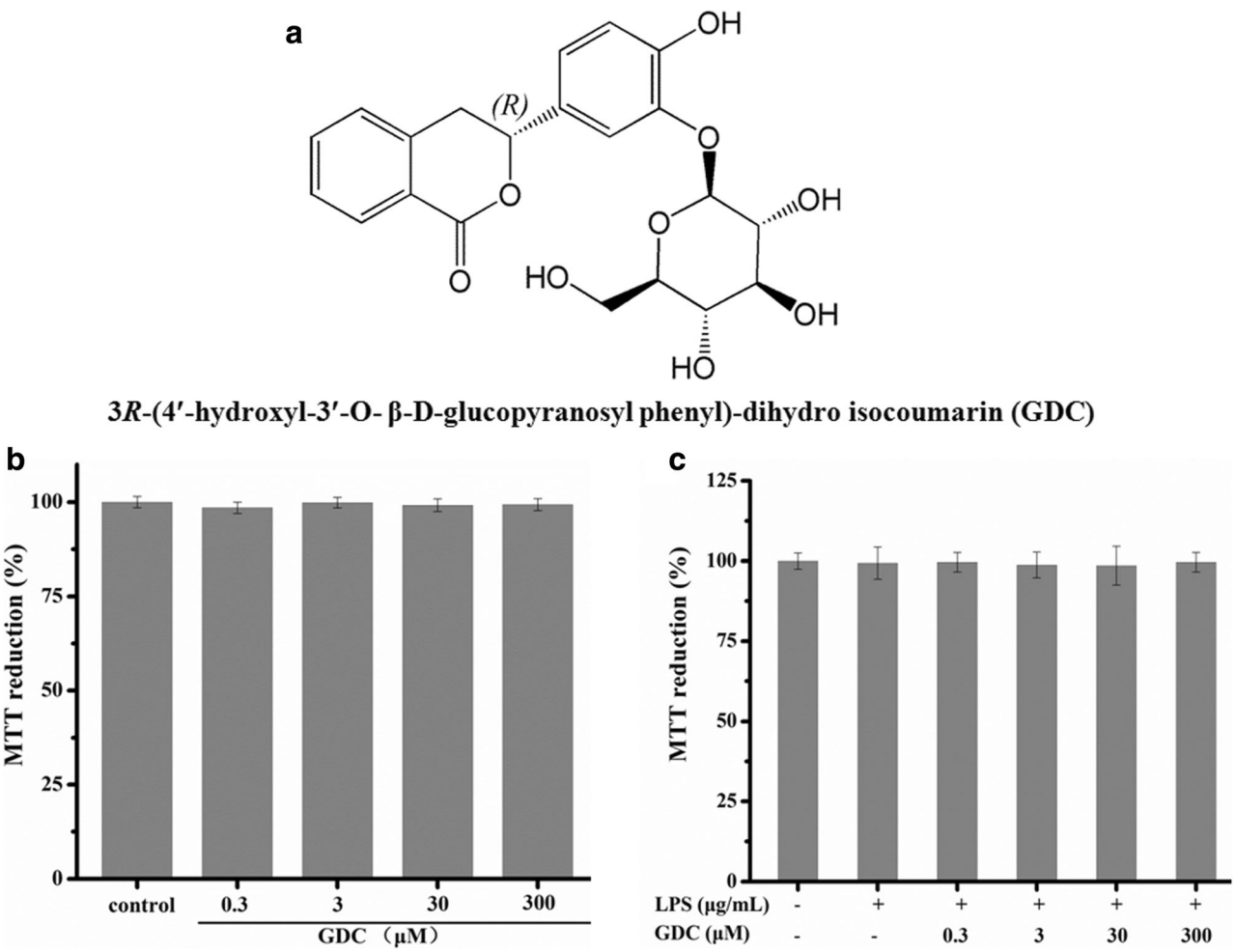

Fig. 1. Effects of GDC on LPS-induced cell viability in FLSs. **a** Chemical structure of 3*R*-(4′-hydroxyl-3′-O- β-D-glucopyranosyl phenyl)-dihydro isocoumarin (GDC). **b** Effects of different concentrations of GDC on the cell viability. **c** Effects of GDC on the cell viability in LPS-treated cells. Cells were treated with indicated concentrations of GDC and incubated in the presence of 1 μg/mL LPS for 24 h. Cell viability was determined by WST solution. Results were expressed as percentages relative to the control group. Data were shown as means ± SD ($n = 6$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* the LPS-treated group.

Array Analysis

The intracellular levels of 33 cytokines were determined using the RayBio® Rat Cytokine Antibody Array 1 per manufacturer's instructions (RayBiotech, Norcross, GA). Briefly, total protein is extracted from cells with ice-cold and the protein concentration is determined by BCA Protein Assay Kit (KangChen KC-430, China). Protein array membranes are blocked in blocking buffer for 30 min and then incubated with samples at room temperature for 1 to 2 h. Samples are then decanted, and membranes are washed with washing buffer. The membranes are washed with washing buffer and then reacted with HRP-conjugated streptavidin (1:1000 dilution) at room temperature for 2 h. Membranes are then washed thoroughly and exposed to detection buffer in the dark before being exposed to X-ray film. After acting to detection buffer, the membranes are exposed to X-ray film, and the image is developed using film scanner. By comparing the signal intensities, relative expression levels of cytokines are made. The intensities of signals are quantified by densitometry. Positive controls are used to normalize the results from different membranes being compared. Fold changes in protein expression are calculated.

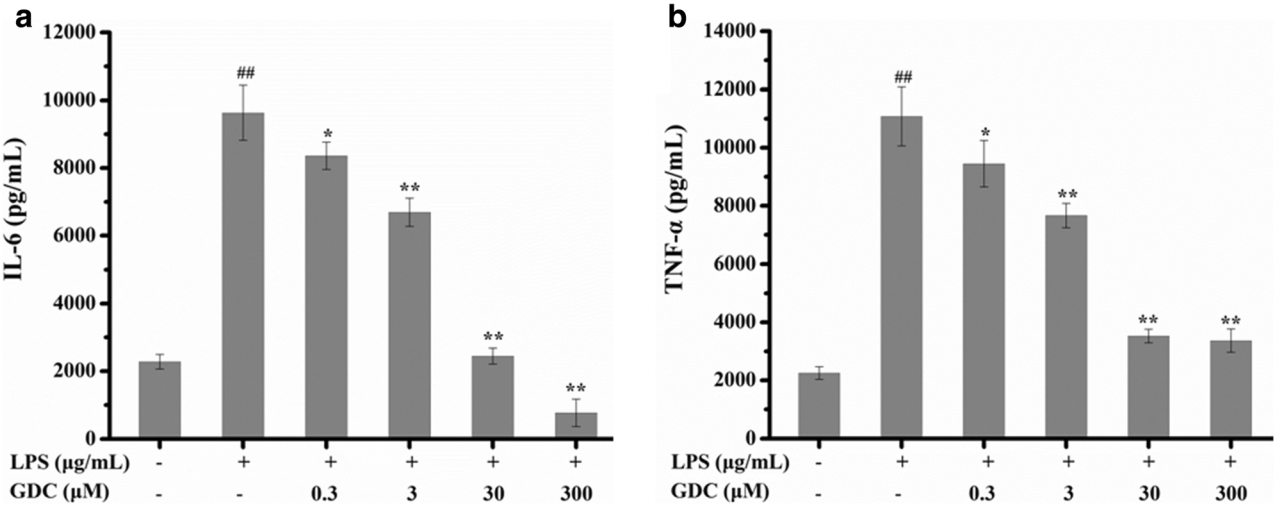

Fig. 2. Effect of GDC on the secretion of IL-6 and TNF-a in LPS-stimulated FLSs. Cells were treated with indicated concentrations of GDC and incubated in the presence of 1 μg/mL LPS for 24 h. Data were shown as means ± SD ($n = 6$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* the LPS-treated group.

Real-Time PCR Analysis

After treatment, cells were harvested and the total RNA was extracted using RNeasy® Mini Kit (QIAGEN). The mRNA was reverse transcribed into cDNA by TransScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China). Quantitative real-time PCR was then carried out on ABI 7900HT real-time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). The sequences of the PCR primers used in this study are listed as follows: IL-6 forward 5′-GAC CAA GAC CAT CCA ACT CAT C-3′ and IL-6 reverse 5′-ACA TTC ATA TTG CCA GTT CTT CGT A-3′; IL-1β forward 5′-ATG ACC TGT TCT TTG AGG CTG AC-3′ and IL-1β reverse 5′-CGA GAT GCT GCT GTG AGA TTT G-3′; TNF-α forward 5′-ATG AGC ACG GAA AGC ATG-3′ and TNF-α reverse 5′-TAC GGG CTT GTC ACT CGA GTT-3′; iNOS forward 5′-CAA GCA CCT TGG AAG AGG AG-3′ and iNOS reverse 5′-AAG GCC AAA CAC AGC ATA CC-3′; GAPDH forward 5′-AGC CCA GAA CAT CAT CCC TG-3′ and GAPDH reverse 5′-AGC CCA GAA CAT CAT CCC TG-3′. The relative transcriptional level of target genes normalized to GAPDH was calculated.

Western Blot Analysis

After treatment, cells were collected and lysed by lysis buffer; then, they were centrifuged at 12,000*g* for 15 min. The supernatant was collected and the protein concentration was determined by the BCA method. Then, protein mixed with loading buffer and incubated in 100 °C for 6 min. Ultimately, samples were analyzed for Western blot analysis with primary antibodies to IKKβ, p-IκBα, IκBα, NF-κBp65, ERK1/2, p-ERK1/2, p-p38, p38, p-JNK, JNK, and β-actin overnight at 4 °C. Finally, they were evaluated using the ECL western detection reagents and the relative expression level of target genes normalized to β-actin was analyzed.

Immunofluorescence Assay

After treatment, cells were fixed with 4% paraformaldehyde followed by permeabilizing in 0.3% Triton X100 and blocking in 5% BSA. Afterward, the coverslips were sequentially incubated with diluted primary antibody against NF-κBp65 (1:400) overnight at 4 °C; secondary antibody

Table 1. The Regulated Differently Expressed Cytokines in FLSs

Name	Column	Row	LPS/control group	GDC/LPS group
Activin A	5	1,2	1.574	0.732
CNTF	12	1,2	1.624	0.738
Fractalkine	2	3,4	1.742	0.534
IFN-gamma	5	3,4	1.833	0.603
IL-1beta	7	3,4	1.965	0.629
IL-4	10	3,4	1.611	0.418
IL-6	11	3,4	1.852	0.535
TIMP-1	12	5,6	1.699	0.646
TNF-alpha	1	7,8	1.654	0.651

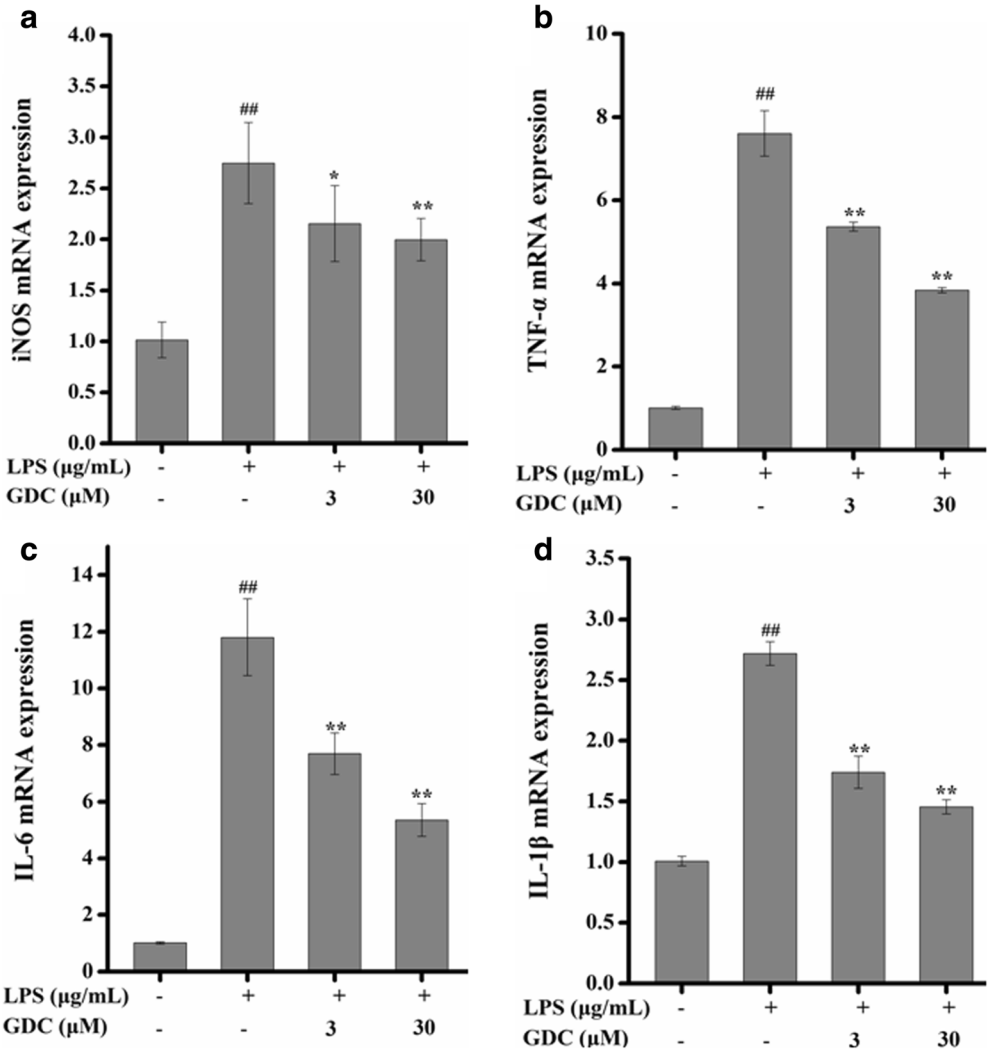

Fig. 3. Effect of GDC on mRNA expression of iNOS, TNF-α, IL-6, and IL-1β in LPS-stimulated FLSs. Cells were treated with indicated concentrations of GDC and incubated in the presence of 1 μg/mL LPS for 24 h. The mRNA levels of iNOS, TNF-α, IL-6, IL-1β, and β-actin were measured using real-time PCR. Results were expressed as relative fold to the control group. Data were shown as means ± SD ($n = 3$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* the LPS-treated group.

(1:1000) was added for 1 h at room temperature and DAPI (50 μg/mL) for 20 min. Image acquisition was achieved using Olympus IX73 fluorescence microscope (Tokyo, Japan).

Statistical Analysis

One-way analysis of variance (ANOVA) (SPSS 20.0 statistical software, Chicago, IL, USA) was used to evaluate multiple group difference. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of GDC on Cell Viability in LPS-Challenged FLSs

As illustrated in Fig. 1, GDC had no significant effect of cell survival on normal synoviocytes (Fig. 1b). Furthermore, 1 μg/mL of LPS, alone or co-treatment with GDC at the concentrations of 0.3–300 μM, did not distinctly affect the cell survival (Fig. 1c).

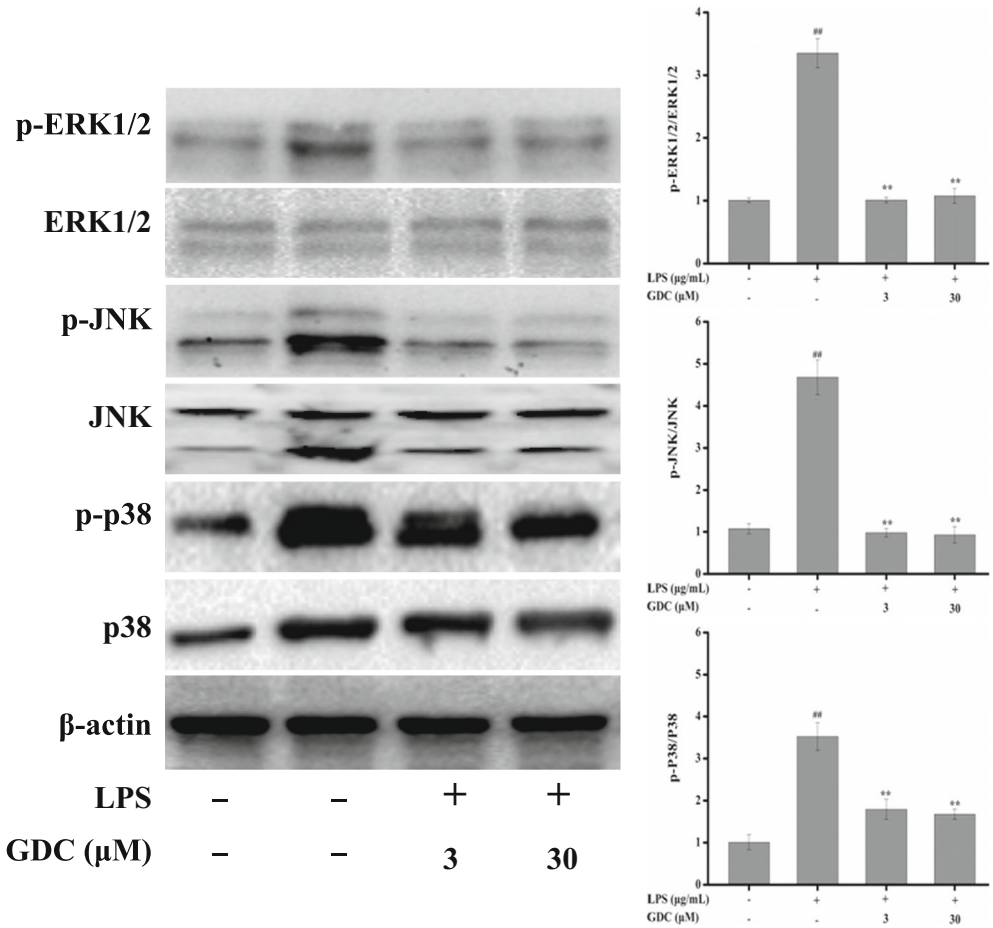

Fig. 4. Effect of GDC on MAPK signaling in LPS-stimulated FLSs. Cells were treated with indicated concentrations of GDC and incubated in the presence of 1 μg/mL LPS for 24 h. Representative gels are shown. Quantification of them was expressed as relative fold to the control group. Data were shown as means ± SD ($n = 3$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* the LPS-treated group.

Effects of GDC on the Secretion of Various Inflammatory Mediators in LPS-Challenged FLSs

As shown in Fig. 2, LPS stimulation led to a robust increase of IL-6 and TNF-a comparing with control group ($P < 0.01$), while they were effectually attenuated by GDC in a dose-dependent manner (0.3–300 μM). Thus, GDC at the concentrations of 3 and 30 μM was adopted to analyze its role in LPS-evoked inflammatory response.

To clarify this point further, cytokine expression profiles were analyzed using a Rat L Series 33: RayBio® Rat Label-based Antibody Array 2, which simultaneously detected the expression levels of 33 rat cytokines. On comparison of GDC (30 μM) group and LPS group, up-regulated differential cytokine was identified by fold change ratio no less than 1.30, and down-regulated differential cytokine was identified by fold change ratio no more than 0.76. Table 1 shows that on comparison to LPS group, activin A, CNTF, fractalkine, IFN-γ, IL-1β, IL-4, IL-6, TIMP-1, TNF-α in GDC group. Taken together, these findings demonstrated that LPS-caused inflammatory response in FLSs was efficiently prevented by GDC treatment.

Effects of GDC on Inflammatory Gene Expression in LPS-Activated FLSs

To further validate the efficiency of GDC on LPS-induced acute inflammatory response in FLSs, the gene expressions of pro-inflammatory cytokines including iNOS, TNF-α, IL-6, and IL-1β were evaluated. As shown

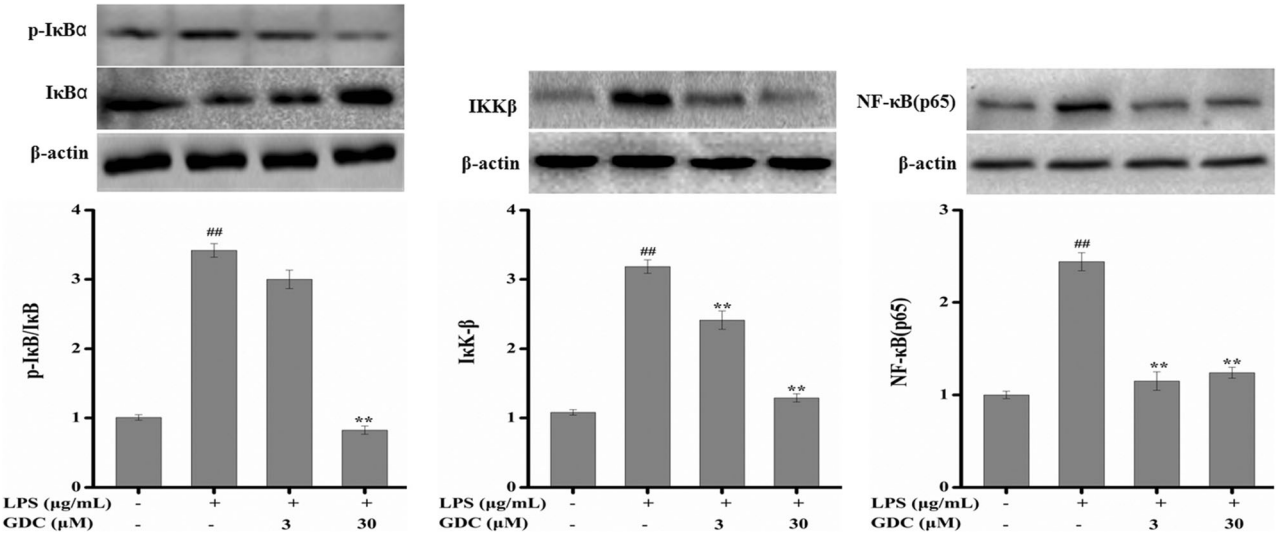

Fig. 5. Effect of GDC on NF-κB signaling in LPS-stimulated FLSs. Cells were treated with indicated concentrations of GDC and incubated in the presence of 1 μg/mL LPS for 24 h. Representative gels are shown. Quantification of them was expressed as relative fold to the control group. Data were shown as means ± SD ($n = 3$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* the LPS-treated group.

in Fig. 3, LPS treatment significantly elevated the mRNA transcript levels of iNOS, TNF-α, IL-6, and IL-1β; these alterations were reversed by GDC treatment.

Effects of GDC on MAPK Pathway and NF-κB Pathway in LPS-Induced FLSs

To further explore the underlying mechanisms of GDC protection, p-ERK1/2 ERK1/2, p-p38, p38, p-JNK, JNK, IKKβ, p-IκBα, IκBα, and NF-κB(p65) were measured by Western blot. As shown in Fig. 4, LPS significantly augmented the phosphorylation of ERK1/2, p38, and JNK; however, they were obviously attenuated by GDC.

In addition, as described in Fig. 5, GDC treatment significantly increased the expression of IκBα and decreased p-IκBα, IkKβ levels, and NF-κB(p65) in the nucleus ($P < 0.01$) compared to LPS group. Furthermore, NF-κB(p65) nuclear translocation from cytoplasm in LPS-induced FLSs (Fig. 6) was observed. GDC treatment significantly inactivated NF-κB(p65) subunit, as indicated by suppressing the nuclear translocation.

DISCUSSION

Several lines of evidence suggest that inflammation plays an important role in the development and progression of RA [21]. Nowadays, targeting the pro-inflammatory mediators secreted by over activated FLSs is still the main research goal. In the present study, it was found that GDC, a new natural isocoumarin, significantly decreased the production of inflammatory mediators and the release of pro-inflammatory cytokines.

It has been proven that pro-inflammatory cytokines, *e.g.*, TNF-α, IL-6, and IL-1β, are overexpressed in the joints in RA and play a major role in the pathogenesis of RA [22–24]. These proinflammatory factors produced by FLSs and immune cells can trigger an inflammatory cascade, which enhance the inflammatory cycle in RA and exacerbate joint erosion consequently. Thus, inhibiting the pro-inflammatory cytokines would be an effective strategy for suppressing inflammation. In the present study, we found that GDC inhibited levels of various inflammatory mediators, including iNOS, TNF-α, IL-6, IL-1β, activin A, CNTF, fractalkine, IFN-γ, IL-4, and TIMP-1 in LPS-stimulated FLSs. It was in consistent with previous studies [25–27]. In addition, our previous study demonstrates that GDC exhibited excellent inhibitory activity on nitric oxide (NO) production induced by lipopolysaccharide (LPS) in mouse macrophage RAW 264.7 cells (10). All these indicated that the GDC might have potential to suppress inflammation in RA.

Inflammatory reactions contribute to RA by multiple signaling pathways. To our knowledge, MAPK and NF-κB signaling pathway are the two most common involved pathways in the inflammatory response [28–30]. NF-κB is an important nuclear transcription factor, and NF-κBp65, one of the NF-κB subunits, is inactivated by binding to inhibitory IκB protein at normal physiological conditions. While when it is stimulated by inflammatory stimuli, such as viruses, bacterial toxins LPS, NF-κB is activated and translocated into the nucleus by phosphorylation of the IκB [28]. Similarly, MAPK signaling pathway including ERK, JNK, and p38 is important in regulating inflammatory mediators. And there are crosstalk between MAPK and NF-κB signaling pathway. It is reported that the phosphorylation of p38 MAPK is a key event upstream of NF-κB activation. Inhibition of p38 MAPK can effectively abolish phosphorylation of IκBα and nuclear translocation of NF-κBp65 [31]. Moreover, phosphorylated IκBα was rapidly degraded by the proteasome including JNK, ERK1/2, and p38 [32]. Activation of MAPK and NF-κB signaling pathway regulates the expression of downstream inflammatory factors, such as TNF, IL-6, and IL-1β, and then participates in a series of inflammatory responses. In this study, we found that GDC treatment significantly inhibited the phosphorylation of ERK, JNK, p38, IκBα in LPS-induced FLSs. Besides this, GDC prevented the nuclear translocation of NF- Bp65 induced by LPS. They suggested that the underlying mechanism of GDC anti-inflammatory activities by inhibiting inflammation response through suppression of the MAPK and NF-κB signaling pathways.

Our study does have some limitations. Studies have reported that glycosides such as ouabain are known as a class to inhibit plasma membrane Na/K ATPase. Ouabain

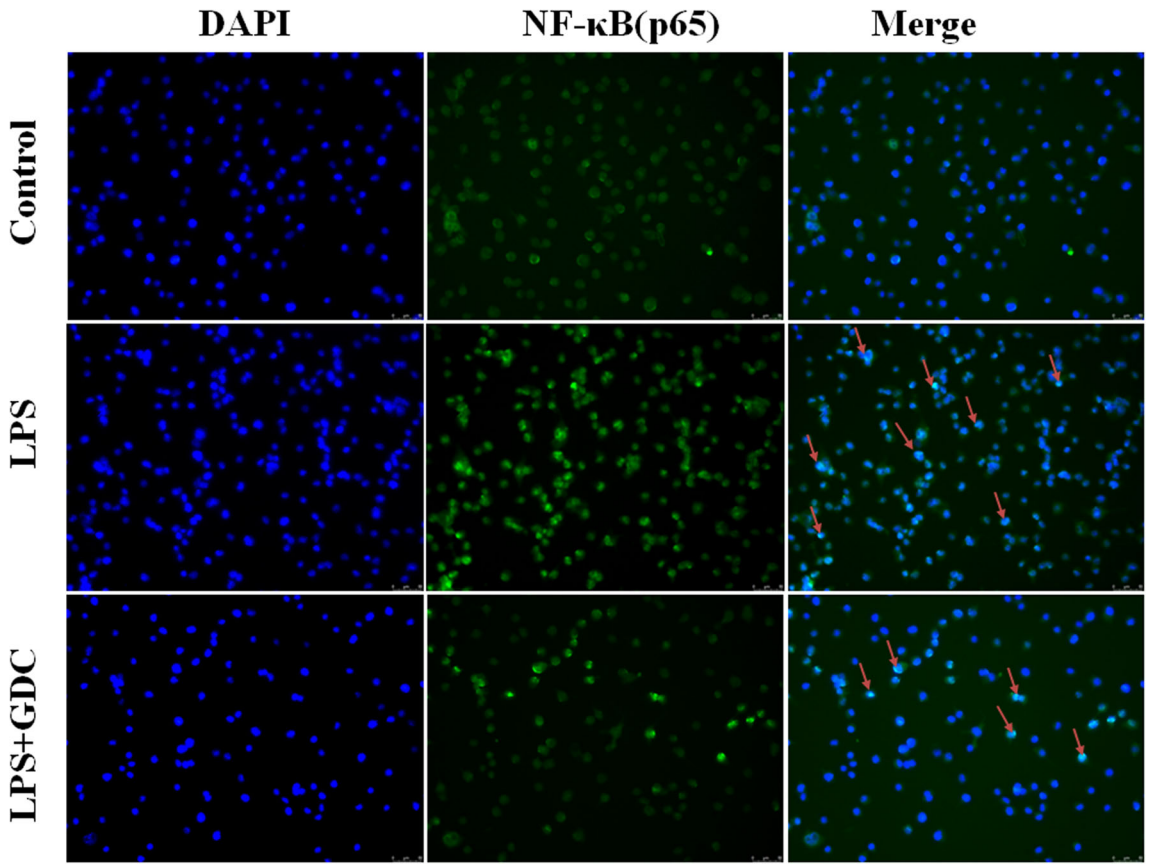

Fig. 6. Effect of GDC on NF-κB nuclear translocation in LPS-stimulated FLSs. FLSs were incubated in the presence to 1 μg/mL LPS with 30 μM GDC for 24 h. Cells were stained with NF-κBp65 antibody (green) and nuclei, DAPI, scale bar = 50 μM. The representative images by fluorescence microscope were shown.

is a well-known example of cardiac glycosides and it is widely used experimentally to inhibit Na/K ATPase [33]. GDC has a similar structure to ouabain which suggests that Na/K ATPase inhibition may contribute to GDC effects. In our follow-up work, we will explore this potential mechanism of GDC to further understand its potential mechanism of action.

CONCLUSIONS

In summary, GDC treatment resulted in significant inhibition of various inflammatory mediators, including iNOS, TNF-α, IL-6, IL-1β, activin A, CNTF, fractalkine, IFN-γ, IL-4, and TIMP-1 in LPS-stimulated FLSs. In addition, the anti-inflammatory activity of GDC was mediated *via* inhibiting of MAPK and NF-κB signaling pathways in LPS-stimulated FLSs.

FUNDING INFORMATION

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interests. The authors declare that they have no conflicts of interest.

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