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ORIGINAL ARTICLE

Narirutin Suppresses M1-Related Chemokine Interferon-Gamma-Inducible Protein-10 Production in Monocyte-Derived M1 Cells via Epigenetic Regulation

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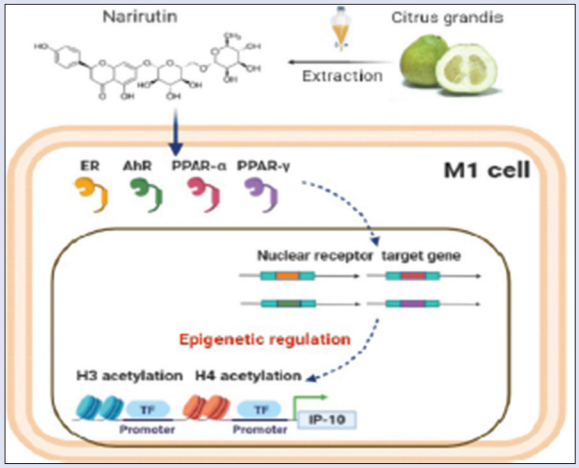
ABSTRACT

Background: Flavonoids are groups of natural phytonutrients found in fruits and vegetables that have recently become popular because of their anti-oxidation and anti-inflammatory ability. Narirutin is a flavanone which has been proven to have anti-inflammation effects, although its fundamental mechanisms are not understood. **Objective:** We try to investigate this anti-inflammatory effect of narirutin in human monocytic THP-1-derived M1 macrophage cells. **Materials and Methods:** To confirm our hypothesis, human THP-1 cells (1×10^6 cells/mL) were initially treated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) for 24 h. The PMA-differentiated THP-1 cells were treated with various levels of narirutin 2 h before lipopolysaccharide stimulation; after that, the cells were cultured for 24–48 h and then examined. The concentration of interferon-gamma-inducible protein-10 (IP-10) was measured using enzyme-linked immunosorbent assay. Epigenetic regulation mechanisms were explored by chromatin immunoprecipitation assay. **Results:** Narirutin significantly suppressed IP-10 production in M1 macrophage cells, and the suppressing effect was partly reversed by the estrogen receptor antagonist, the aryl-hydrocarbon receptor antagonist, the peroxisome proliferator-activated receptor (PPAR)-α antagonist, and the PPAR-γ antagonist. We also found that narirutin-induced IP-10 suppression can be modulated by both histone H3 and H4 acetylation. **Conclusion:** Our study suggests the potential of narirutin for the treatment of inflammatory disease by suppressing IP-10.

Key words: Flavonoid, inflammation, interferon-gamma-inducible protein-10, M1 macrophage cell, narirutin

SUMMARY

- Flavonoid narirutin has suppressive effects on IP-10 in monocyte-derived M1 macrophage through nuclear receptors including ER, AhR, PPAR-α and PPAR-γ receptors; suppressive epigenetic regulation viaH3 and H4 acetylation was also observed.



Abbreviations used: PMA: Phorbol 12-myristate 13-acetate; LPS: Lipopolysaccharide; IP-10: Interferon-gamma-inducible protein-10; DMSO: Dimethyl sulfoxide; PBS: Phosphate-buffered saline; ELISA: Enzyme-linked immunosorbent assay; ChIP: Chromatin immunoprecipitation; ER: Estrogen receptor; AhR: Aryl-hydrocarbon receptor; PPAR: Peroxisome proliferator-activated receptor; AA: Anacardic acid; MDC: Macrophage-derived chemokine; IFN-γ: Interferon-gamma; TNF-α: Tumor necrosis factor-alpha; PCR: Polymerase chain reaction; CXCR3: C-X-C motif chemokine receptor 3; HAT: Histone acetyltransferase.

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INTRODUCTION

Inflammation is an innate immune response induced by cytokines and chemokines, which are released by immune cells in response to harmful stimuli or the need for a healing process.[1] However, inflammation is also a double-edged sword in human bodies, causing slight inflammatory symptoms or severe diseases. Although adequate inflammation is the innate defensive system against pathogens, inappropriate chronic inflammation may lead to tissue damage.

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Monocytes and macrophages have been considered major immune effector cells due to their important functions, including presenting antigens, inducing phagocytosis, and immunomodulating process through cytokines. Therefore, macrophages have a critical role in inflammation regulation by both initiating and deactivating the inflammation process.[2]

To our knowledge, macrophages can mediate Th1 with Th2 balance using Th1-related chemokines (for example, interferon-gamma-inducible protein-10 [IP-10]) or Th2-related chemokines (for example, macrophage-derived chemokine). Chemokines consist of small cytokines that can affect the migration and location of immune cells in inflammation responses.[3] In response to interferon-gamma (IFN-γ) or TNF-α by recruited Th1 lymphocytes, IP-10 is produced by various differential cells including T-lymphocytes, macrophages, endothelial cells, and fibroblasts.[4] Thus, IP-10 has become popular in investigating inflammatory response and chemoattraction in recent immunological research.

Flavonoids are common phytochemicals extensively existing in vegetables and fruits; they are classified into six groups according to their basic skeleton, including flavones, isoflavones, flavanones, anthocyanidins, flavonols, and flavanols.[5] Narirutin is a flavanone that is plentiful in citrus fruits, including grapefruits and oranges. Multiple benefits of flavonoids have been discovered, such as anti-nociception, anti-oxidation, anti-inflammation, anti-carcinogenesis, anti-allergy, and immunological modulation.[6,7]

Evidence has established an important role for the benefits of flavonoids in reducing allergic expression by suppressing chemical mediators and Th2-type cytokines.[8] Nevertheless, few reports are discussing the association between flavonoids with allergy, and their definite role in the immune response remains unexplored.

Previous studies have revealed that narirutin has the anti-inflammatory ability *in vivo* with *in vitro* at animal experiments.[9] However, studies related to the underlying pathways of the anti-allergic and anti-inflammatory ability in narirutin are rare. Here, we hypothesized that narirutin may inhibit inflammation by modulating IP-10 production. In this work, we investigated the effect of flavonoid narirutin on its anti-inflammatory responses in monocyte-derived M1 macrophage and further explored potential cellular mechanisms, including epigenetic activity.

MATERIALS AND METHODS

Chemicals

Narirutin SMB 00321 (>98% purity), phorbol 12-myristate 13-acetate (PMA), IFN-γ, lipopolysaccharide (LPS), estrogen receptor (ER) antagonist ICI 182780 (>98% purity), aryl-hydrocarbon receptor (AhR) antagonist CH-223191 (>98% purity), peroxisome proliferator-activated receptor (PPAR)-α antagonist GW 6471 (>98% purity), PPAR-γ antagonist GW 9662 (>98% purity), anacardic acid A7236 (> 98% purity), and IP-10 (Human CXCL10/IP-10 Duoset enzyme-linked immunosorbent assay [ELISA]) were obtained from Sigma-Aldrich Chemical Company (Saint Louis, USA). Antibodies for acetyl-Histone H3 (06-559) and acetyl-Histone H4 (06-866) were obtained from Millipore Company (Massachusetts, USA).

Experimental design

Cell preparation

An RPMI-640 medium (from Sigma-Aldrich) augmented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum was prepared first. Then, human monocytic THP-1 cells (American Type Culture Collection, Rockville, USA) were incubated with the above medium in a humidified incubator containing 5% CO_2 at 37°C. After centrifuging with resuspending in 12-well flat-bottomed plate with new media, these cells were incubated at 2×10^5/mL concentration for experiment use. The THP-1 cells were pretreated with PMA, IFN-γ, and LPS all at 20 ng/mL for 72 h as an inflammatory challenge for M1 macrophages. The supernatant was further analyzed for the cytokine profile.

Cell viability assay (XTT assay)

Narirutin was dissolved in DMSO, and then, the above solution was used to dilute with PBS. THP-1-derived M1 macrophages were pretreated with several levels of narirutin (10^{-9} to 10^{-5} M) in 96-well plates for 24 and 48 h. XTT activation solution was blended with the reagent in a 1:10 dilution rate. Each well and plate was added with the above 10 μL reaction solution and then was incubated 1 h before use. ELISA reader (Bio-Rad Benchmark Plus microplate spectrophotometer) was used to measure the absorbance of the sample to calculate the cell viability. The ELISA reader was reading at a wavelength of 450 nm and 600 nm as reference. The cell viability was recorded as a percentage of control and assessed using the average value of the content in 6 wells. CytoScan WST-1 Cell Proliferation Assay (G-Biosciences, Maryland Heights, USA) was used to exam cell viability level, and the results are demonstrated as a relative percentage of control value.

Enzyme-linked immunosorbent assay

M1 macrophages were pretreated with several levels of narirutin (10^{-6} with 10^{-5} M) or without narirutin for 2 h, and then, LPS 0.2 μg/mL was used to stimulate the above cells. ELISA analysis was done by collecting cell supernatant samples which were harvested 24 h after LPS stimulation. To identify involved intracellular signaling pathways, M1 cells were treated with ER antagonist ICI 182780, AhR antagonist CH-223191, PPAR-α antagonist GW 6471, PPAR-γ antagonist GW 9662, and AA (a histone acetyltransferase inhibitor) A7236 1 h before narirutin treatment. IP-10 levels in the cell supernatants were expressed using ELISA assay systems with kits (R&D Systems Inc., Minneapolis, USA). Dynatech MRX plate reader was used to measure the absorbance in each well, with wavelength set at 450 nm with 550 nm. Revelation software (Dynatech Laboratories Ltd., Virginia, USA.) was used to calculate the results. All assays were done with instructions by the manufacturer.

Chromatin immunoprecipitation assay

To investigate epigenetic regulation, the chromatin immunoprecipitation (ChIP) assay was done as our previous work with minor modifications.[10,11] M1 macrophages (1×10^6/mL) were pretreated with several levels of narirutin (10^{-7} to 10^{-5} M) or without narirutin 2 h before 1-h LPS (0.2 μg/mL) stimulation. 1% formaldehyde was added to stimulated macrophages for 10 min at room temperature first, followed by DNA sonication and ChIP with antibodies for acetyl-histone H3 (06-559) and acetyl-histone H4 (06-866) 2 h before LPS stimulation separately. Protein A slurry (Invitrogen) was used to collect immune complexes, and TaqMan SDS 7900 HT was used for DNA reverse cross-linking extraction and quantification.

PCR primers specific to the proximal promoter regions of IP-10 gene were applied to identify the immune complexes associated with DNA. Specific primers above were used as follows: 5'-GAGGGAAATTCCGTAACTTGG-3' (sense) and 5'-TCAGAAAACGTGGGGCTAGT-3' (antisense), including the

following subregions (+9/−172).[12] ABI 7700 TaqMan thermocycler (Applied Biosystems, Thermo Fisher Scientific, USA) was utilized to perform PCRs. The relative intensity of expanded products was quantified comparing to input DNAs.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software, version 5.01, La Jolla, USA) was utilized for data analysis, and these results were expressed as mean ± standard deviation. All statistical comparisons were done by one-way analysis of variance, while multiple comparisons were done by the Student–Newman–Keuls test. $P < 0.05$ was statistically significant.

RESULTS

Narirutin suppressed lipopolysaccharide-stimulated interferon-gamma-inducible protein-10 production in M1 macrophage

We found that stimulating with LPS results increased the expression of IP-10 in M1 macrophage, and the effect was significantly reversed by narirutin in dose- and time-dependent trend. Figure 1a and b reveals that narirutin at concentrations of 10^{-8} to 10^{-6} M suppressed IP-10 expression after 24 and 48 h of LPS stimulation.

Narirutin showed no toxic effect on the lipopolysaccharide-stimulated M1 macrophage

To investigate the influence of narirutin on the viability of M1 macrophage cells, we performed XTT assay with 24-h and 48-h incubation to increase concentrations of narirutin. Narirutin did not affect the viability of M1 macrophage after LPS stimulation at both 24 and 48 h [Figure 1c and d]. The result showed that narirutin has no cytotoxicity on LPS-stimulated M1 macrophage at the concentration we used (10^{-9} to 10^{-5} M) in 24 and 48 h after narirutin treatment.

Narirutin suppressed lipopolysaccharide-stimulated interferon-gamma-inducible protein-10 expression via the estrogen receptor, aryl-hydrocarbon receptor, peroxisome proliferator-activated receptor-α, and peroxisome proliferator-activated receptor-γ

To our knowledge, many botanical compositions have physiological effects by modulating nuclear receptors including the estrogen receptor (ER), the AhR, and PPARs. We further investigate whether the suppression effects of narirutin was mediated via these receptors. In

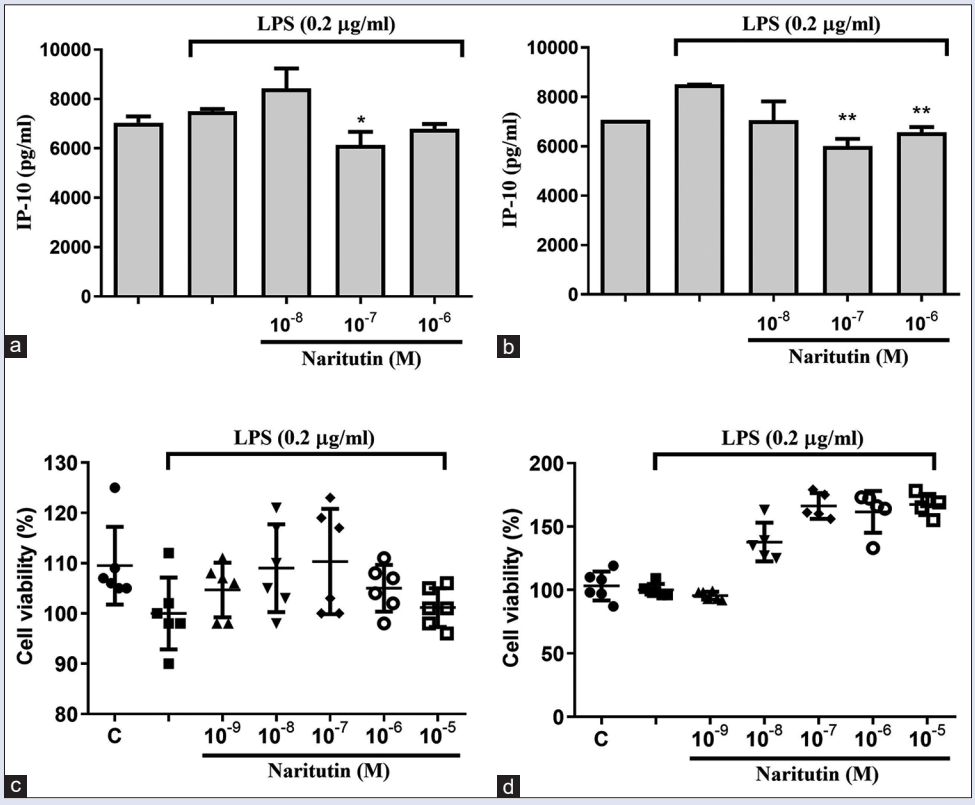

Figure 1: Effect of narirutin on the production of interferon-gamma-inducible protein-10 in M1 macrophage cells. Narirutin (10^{-7} M and 10^{-6} M) suppressed interferon-gamma-inducible protein-10 production at (a) 24 h and (b) 48 h after lipopolysaccharide stimulation in M1 macrophage cells. All cells were pretreated with narirutin at different concentrations 2 h before lipopolysaccharide addition. Data are presented as mean ± standard deviation values of triplicate measurements of three independent experiments in $^{*}P < 0.05$ and $^{**}P < 0.01$ between groups with and without narirutin pretreatment. Narirutin had no effect on the viability of M1 macrophage cells at 24 h and 48 h after lipopolysaccharide stimulation (c and d). The cell viability in each group was expressed as a percentage of the control group. Six independent experiments were performed

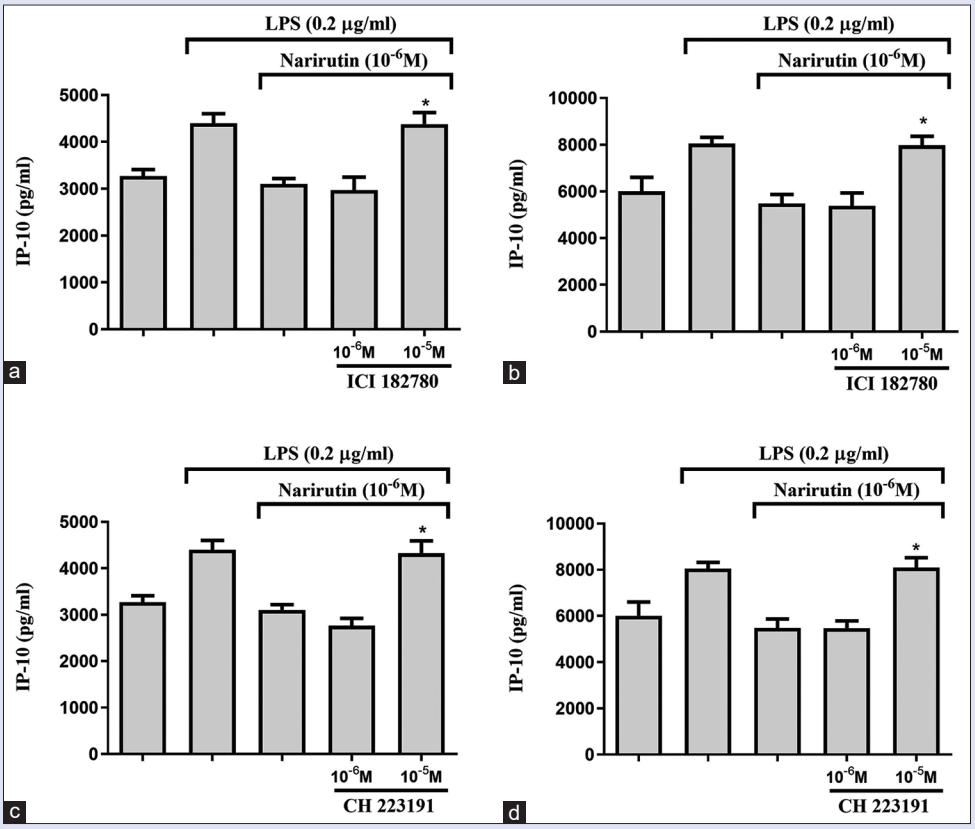

Figure 2: The inhibition of interferon-gamma-inducible protein-10 production in M1 macrophages by narirutin was reversed by the ER antagonist (ICI 182780) and aryl-hydrocarbon receptor antagonist (CH 223191) in 24 h (a and c) or 48 h (b and d) after lipopolysaccharide plus narirutin pretreatment. Data are presented as mean ± standard deviation values of triplicate measurements of three independent experiments. *$P < 0.05$ between groups with and without ER antagonist/aryl-hydrocarbon receptor antagonist treatment

Figure 2, ER antagonist (ICI 182780) and AhR antagonist (CH223191) reversed the suppressive effect of narirutin on LPS-stimulated IP-10 production at high concentration (10^{-5} M) [Figure 2a-d]. Likewise, both PPAR-α and PPAR-γ antagonists reversed the suppressive effect of narirutin on LPS-stimulated IP-10 production at high concentration (10^{-5} M) [Figure 3a-d].

Lipopolysaccharide-stimulated interferon-gamma-inducible protein-10 expression in M1 macrophage was reversed by anacardic acid pretreatment

Anacardic acid is a 6-alkyl salicylic acid that inhibits the histone acetyltransferase (HAT) activity and can suppress inflammation through modulating NF-κB activation.[13] In this study, we suspected that LPS-stimulated IP-10 production in M1 macrophage is relevant to epigenetic regulation. Therefore, we performed the ChIP assay, and our result revealed that IP-10 production was suppressed by anacardic acid at concentrations of 10^{-6} and 10^{-5} M, which are compatible with the results of previous research [Figure 4a and b].

Narirutin suppressed lipopolysaccharide-stimulated interferon-gamma-inducible protein-10 production via histone acetylation in M1 macrophage

Epigenetic regulation is closely associated with pro-inflammatory gene expression in human monocytes.[14] Besides, our previous works revealed that IP-10 production in human monocytes may be regulated through histone acetylation.[15] Based on this conclusion, we next investigated whether the suppression effects of narirutin were mediated through histone acetylation. ChIP assay was done, which showed that the suppressive effect of narirutin on H3 and H4 acetylation was mediated by IP-10 promoter in M1 macrophage [Figure 5a and b]. The data implied that narirutin suppressed IP 10 production partially through regulation of LPS stimulated histones H3 and H4 acetylation.

DISCUSSION

In this work, we presented the effects of narirutin on the production of inflammation relevant chemokine IP-10 in LPS-stimulated human macrophages. Besides, we also reported evidence for the detailed activating pathway of narirutin, which included nuclear receptors with epigenetic regulation for suppressing IP-10 production in M1 macrophages.

Flavonoid compounds have anti-inflammation ability according to recent studies; flavonoids such as myricetin, kaempferol, quercetin, and luteolin can inhibit the degranulation of mast cells and IgE-mediated cytokines; therefore, they can reduce the activation of allergic reactions.[7,8,16] However, narirutin has been found to suppress airway inflammation in allergic mice, which implies that the mechanism of the anti-inflammatory effect of narirutin is associated with downgrading IL-4 and IgE production in the allergic eosinophilic airway of murine.[9] Similar research on the beneficial effects of natural flavonoids has been published in the literature.[10,11,17,18]

Macrophages are divided into two clusters: classically activated macrophages (M1 macrophages) and alternatively activated

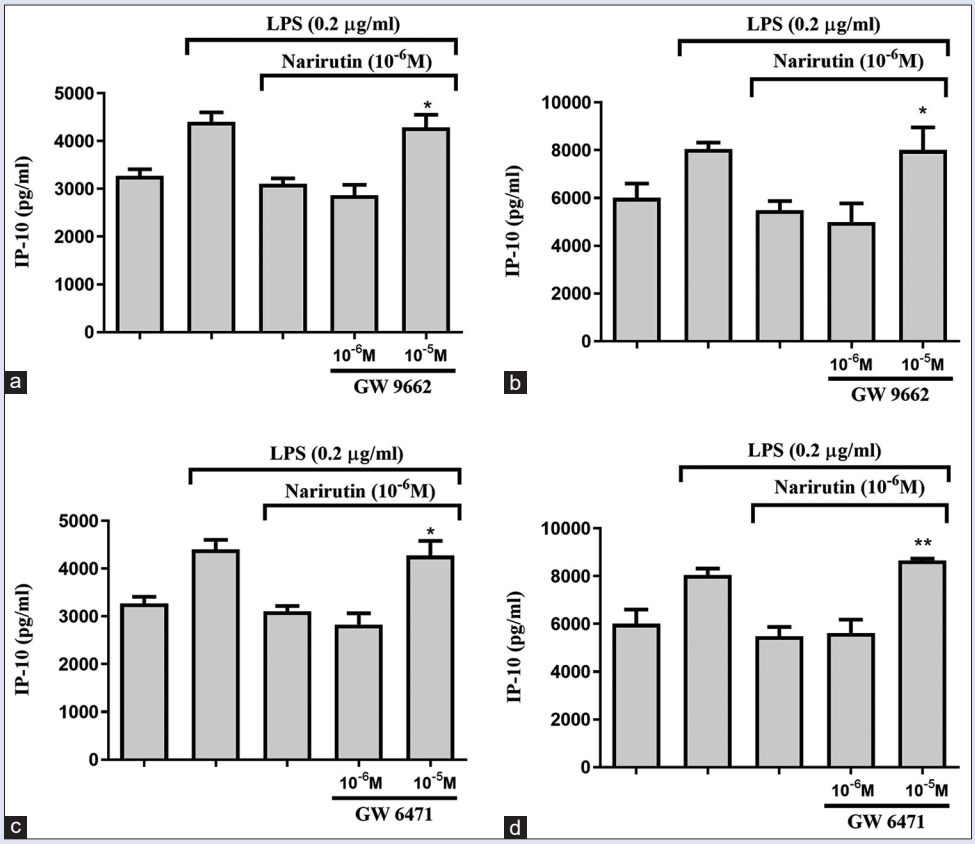

Figure 3: The inhibition of interferon-gamma-inducible protein-10 production in M1 macrophages by narirutin was partly reversed by peroxisome proliferator-activated receptor-α and peroxisome proliferator-activated receptor-γ antagonist in 24 h (a and c) or 48 h (b and d) after lipopolysaccharide pretreatment. Moreover, this regulation effect of interferon-gamma-inducible protein-10 in narirutin could be regulated through both peroxisome proliferator-activated receptor-α and peroxisome proliferator-activated receptor-γ at a higher level (10^{-5} M). Data are presented as mean ± standard deviation values of triplicate measurements of three independent experiments. **P* < 0.05 and ***P* < 0.01 between groups with and without peroxisome proliferator-activated receptor-α antagonist/peroxisome proliferator-activated receptor-γ antagonist treatment

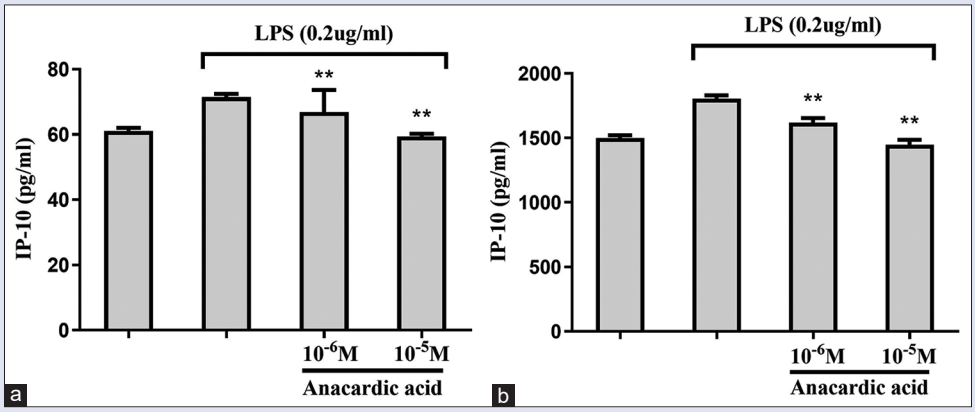

Figure 4: Lipopolysaccharide-stimulated interferon-gamma-inducible protein-10 production in monocyte-derived M1 cells was partly reversed by anacardic acid. Anacardic acid (10^{-7} M and 10^{-6} M) suppressed interferon-gamma-inducible protein-10 production in the M1 cell at (a) 24 h and (b) 48 h after lipopolysaccharide stimulation. Data are presented as mean ± standard deviation values of triplicate measurements of three independent experiments. **P* < 0.05 and ***P* < 0.01 between groups with and without anacardic acid treatment

macrophages (M2a, M2b, and M2c macrophages) according to their function and differentiation.[19] The Th1 response can be induced by exposure to bacterial lipopolysaccharide, where Th1 cells with M1 macrophages are activated, which can produce TNF-α, IFN-γ, and IL-12. These cytokines regulate cell-mediated immunity, including a phagocyte-dependent immune reaction to eliminate the intracellular pathogen and cause further autoimmune responses. Th1 cells produce IFN-γ to promote M1 macrophage activation, while M1 macrophages secrete IL-12 to further recruit Th1 cells.[20] Thus, in human adaptive inflammation responses, M1 macrophages play a critical role in

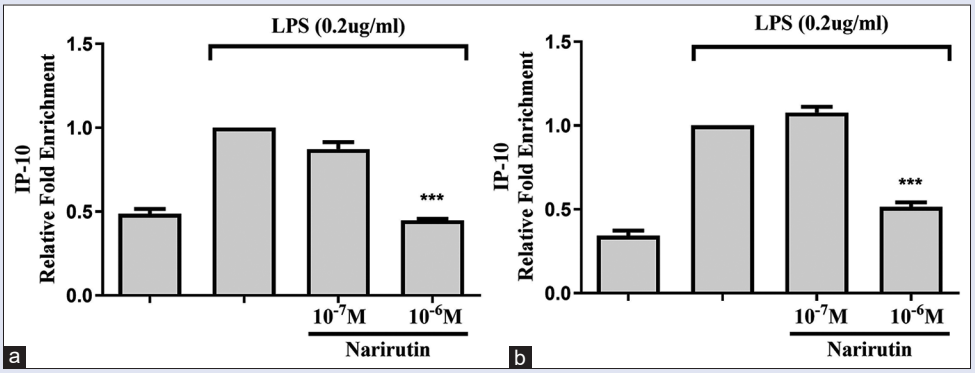

Figure 5: Histone acetylation was involved in the suppressive effects of narirutin on interferon-gamma-inducible protein-10 production in monocyte-derived M1 cells. Narirutin at 10^{-6} M suppressed lipopolysaccharide-induced acetylation of histone H3 (a) and H4 (b) acetylation in the interferon-gamma-inducible protein-10 promoter region in M1 cells. Data are presented as mean ± standard deviation values of triplicate measurements of three independent experiments. ***$P < 0.001$ for between groups with and without narirutin pretreatment

defending the host and are involved in immune homeostasis balanced by Th1 responses after exposure to antigens.[21]

Recent research has shown that IP-10 has an important role in chemoattraction for T-cells and macrophages in Th1-mediated inflammatory diseases by binding to its receptor CXCR3 and promoting the recruitment of Th1 cells to create an amplification feedback loop. The important role of IP-10 has been clearly illustrated in chronic Th1 inflammatory diseases.[22,23] Therefore, targeting IP-10 production is another potential approach for inflammation control. In this work, we found that narirutin suppressed IP-10 expression in THP-1-derived M1 macrophages. These findings presented persuasive proof that narirutin has a suppressive effect by regulating the production of Th1-related chemokines in M1 macrophages.

The phytogenic effects of flavonoids can be multiple nuclear receptor modulators; naringenin (a flavanone same type as narirutin) had ER, PPAR-α, and PPAR-γ receptor affinity. Other flavonoids such as apigenin only had PPAR-γ receptor affinity and quercetin had only PPAR-α and PAR-γ receptor affinities in past studies.[22] Many phytoestrogens are members of the flavonoid family, and some share a similar character with selective estrogen receptor modulators (SERMs) with lower potency.[24] These findings are also compatible with our findings which suggest that the anti-inflammatory effects of narirutin may be attributed to nuclear receptor regulation.

The epigenetic regulation of IP-10 has been previously illustrated by Kuo *et al.*, which has involved the suppression of H3 acetylation and the trimethylation of H3K4 with H3K36 in IP-10 promoter area; our results implied that narirutin may inhibit IP-10 production by suppressing acetylation of H3 promoter region.[15,25] Works of literature involving histone acetylation in M1 macrophages are relatively rare. Nevertheless, our study implied that narirutin may regulate histone acetylation by suppressing IP-10 production.

CONCLUSION

We described that narirutin may suppress IP-10 production in M1 macrophage cells through nuclear receptors including ERs, AhRs, PPAR-α and PPAR-γ receptors at different degrees. Besides, we demonstrated that the epigenetic regulation of histones via H3 acetylation in IP-10 promoter area may be related to the suppressive effect of narirutin on LPS-stimulated IP-10 expression. These findings revealed that narirutin may adjust anti-inflammation effects through suppressing nuclear receptors with histone acetylation. Further animal experiments with human trials should be conducted to clarify the clinical advantages of flavonoids toward inflammation diseases. Our study increases the knowledge of the possible therapeutic mechanisms in narirutin, which might be an alternative strategy for further clinical treatment against inflammation reactions by suppressing IP-10.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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