7-Methoxyisoflavone Suppresses Vascular Endothelial Inflammation by Inhibiting the NF-κB Signaling Pathway

Xinyu Zhu
Wuhan Union Hospital
Cheng Wang
Wuhan Union Hospital
Kai Huang
Wuhan Union Hospital
Minglu Liang (liangml@hust.edu.cn)
Wuhan Union Hospital https://orcid.org/0000-0002-4026-951X

Original Article

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Abstract

Objective: This study aimed to investigate the effect of 7-Methoxyisoflavone (7-Mif) on the inflammation.

Materials and Methods: The cytotoxic effect of 7-Mif on HUVECs was tested by CCK8 kit. The expression of intracellular adhesion protein-1 (ICAM-1) and vascular cell adhesion protein-1 (VCAM-1) were tested by RT-qPCR and Western blot. Monocyte-endothelial cells adhesion assays was performed by Vybrant™ DiD Cell-Labeling Solution. The NF-κB activation was tested by Western blot and Immunofluorescence. And LPS induced animal experiment was performed.

Results: 7-Mif show no cytotoxicity on HUVECs. Pre-treated with 7-Mif can significantly reduce ICAM-1 and VCAM-1 expression both on mRNA and protein level, and further inhibit the monocyte-endothelial cells adhesion. On the mechanism, our results showed that 7-Mif can reverse IL-1β induced NF-κB activation and p65 translocation to nucleus, therefore, inhibit inflammation of endothelial cells. In addition, we confirmed that 7-Mif can attenuate the inflammatory injury induced by LPS in mice.

Conclusion: Here, we studied the inhibitory effect of 7-Mif on the endothelium inflammation by suppressing the expression of inflammatory gene and monocyte adhesion. Our data illustrated that 7-Mif could be positively regulate the process of endothelium inflammation.

1. Introduction

Inflammation is a response of body to eradicate the stimulus like infectious microbes damaged tissues. In the initial stage, the resident leukocytes (such as macrophages) sense those stimulus, and secrete multiple paracrine molecules[7]. Endothelial cells, which constitute the intima and in direct contact with the blood flow, therefor are activated by those cytokines and molecules with increased vascular permeability and upregulated endothelial adhesion molecules like ICAM-1 and VCAM-1[12]. Then leukocytes in the circulation such as neutrophils are recruited, excited, and eventually reach to the inflamed area, causing a positive loop feedback[8]. Once the stimulus were exterminated, the inflammatory response will be dampened. On the contrary, persistent stimulus can lead to chronic inflammation, and dysfunction of endothelial cells and monocytes contributes to various inflammatory diseases like atherosclerosis[1], inflammatory bowel disease and multiple sclerosis[11]. Considering the vital role of ECs in inflammatory response, it's potential to be therapeutic target of those chronic diseases by inhibiting the ECs' inflammation.

7-Mif is a synthetic isoflavone whose effect on disease has not been studied yet. Its' contracture is shown in Fig. 1A. Based on previous studies about other isoflavone derivative revealing their inhibitory effect on inflammation and related diseases[16], we believe that 7-Mif has a potential to be beneficial effect on endothelial inflammation, and we demonstrated it in this study.

2. Materials And Methods

2.1 Reagents

7-Mif (MCE, Shanghai, China) was dissolved to 3 various concentrations (5 mM, 10 mM, and 50 mM) in dimethyl sulfoxide (DMSO). The eventual proportion of DMSO did not exceed 0.1%, and vehicle control was treated with DMSO only. Interleukin 1β (IL-1β) was dissolved in ultrafiltered water and the final concentration of it was 10 ng/ ml. Lipopolysaccharide (LPS) were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China).

2.2 Cell cultures

Primary human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell medium (No. 1001, ScienCell, USA), supplemented with 5% fetal bovine serum, 1% endothelial cell growth supplement and 1% penicillin-streptomycin solution in 37°C and 5% CO_2. Human monocyte THP-1 (THP-1) cell lines were obtained from American Type Culture Collection (ATCC, USA).

2.3 Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted with TRIzol reagent (TaKaRa) from HUVECs. Reverse transcription was subsequently performed with one microgram of total RNA and the RNA PCR kit (TaKaRa). Then the cDNA was used for quantitative real-time PCR with an ABI Prism 7900 sequence detection system (Applied Biosystems) and SYBR green I (TaKaRa). The following primers were used: 18S (internal control): sense, 5'-GCACCACCACCCACGGAATCG-3' and antisense, 5'-TTGACGGAAGGGCACCACCAG-3'; human VCAM-1: sense, 5'-GGGAAGATGGTCGTGATCCTT-3' and antisense, 5'-TCTGGGGTGGTCTCGATTTTA-3'; human ICAM-1: sense, 5'-ATGGCAACGACTCCTTCTCG-3', and antisense, 5'-GCCGGAAAGCTGTAGATGGT-3'.

2.4 Monocyte adhesion assay

After HUVECs were seeded in a 6-well cell culture plate for 24 h, they were treated with 7-Mif (50 μM) or vehicle (DMSO) for 15 minutes, followed by IL-1β (10 ng/mL) or PBS for 4 hours. Labeling with 5 μM Vybrant DiD was performed for 30 minutes according to manufactures' instruction. Labeled THP-1 cells were then added to medium containing HUVECs and incubated for 30 min. THP-1 cells that didn't bound to HUVECs were removed by washing with PBS. The amount of adhering green fluorescent THP-1 cells were measured with fluorescence microscopy (Olympus, USA).

2.5 Dual Luciferase Reporter Gene Assay

293T cells grew in a 24-well plate till 80% confluence. Then 293T cells were pretreated with 7-Mif (50 μM) for 15 min. The corresponding plasmid was used to transfect the 293T cells. 24 hours later, the cells were harvested and the Dual Luciferase Reporter Gene Assay Kit (Beyotime, RG027) was used to detect corresponding indicators according to the instructions.

2.6 Western blot

Proteins were isolated from cells and total amount of protein was measured by the BCA protein assay kit (Pierce, Rockford, IL, USA). Then protein was resolved in sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) gel and then, transferred to nitrocellulose membranes. 5% nonfat milk solved in tris-buffered saline–Tween 20 (TBST) was used for membranes blocking for 60 minutes at room temperature. After that, the membranes were incubated with primary antibody (1:1000) (anti-ICAM-1 [Proteintech], anti-VCAM-1 [Proteintech], anti-β-tubulin [Abcam], anti-NF-κB-p65 [Cell Signaling], anti-phospho-NF-κB-p65 [Cell Signaling], anti-IκBα [Cell Signaling], or anti-phospho-IκBα [Cell Signaling]) at 4°C overnight. Membranes were washed by TBST for 3 times and incubated with a peroxidase-conjugated secondary antibody for 2 hours at room temperature. After TBST washing, specific bands were analyzed by chemiluminescence assays (ECL detection reagents; Pierce) and X-ray film.

2.7 Nuclear and cytoplasmic proteins extracts

Nuclear protein and cytoplasmic protein extraction kit (Beyotime, P0027) was utilized for muclear and cytoplasmic protein extraction in accordance with instructions. Histone Lamin B1 (ABclonal) and Actin (Proteintech) acted as internal control for nuclear protein and cytoplasmic protein respectively.

2.8 Immunofluorescence

HUVECs were excited with IL-1β (10 ng/ml) or PBS after treated with7-Mif (50 μM) or vehicle (DMSO) for 2h, then HUVECs were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes and followed with 1% Triton X-100 for 10 min at room temperature. After washing 3times with PBS, samples were blocked with 1% BSA for 1 h. Then, cells were incubated with anti-P65 antibody at 4 ℃ overnight. After washed again, cells were incubated at room temperature for 60 minutes with Alexa Fluor 488 conjugated anti-rabbit antibody (ThermoFisher Scientific). DNA binding dye 4,6-diamino-2- phenylindole (DAPI) was used for Nuclei staining for 10 min. Images were obtained with fluorescence scanner (Olympus, USA) and Olympus cellSens version 1.5 imaging software.

2.9 Cell viability assay

Cell viability was analyzed by using the CCK8 kit (Biyuntian, China), according to the instructions manual. Briefly, 5000 HUVECs were seeded in 96-well plates for 12 h and subsequently administrated with 7-Mif (1 μM, 5 μM, 10 μM, 50 μM and 100 μM) or vehicle (DMSO) for 4 h. Cells were incubated with CCK8 for 2 h and the absorbance at 450 nm was detected using a microplate reader (Multiscan FC, ThermoFisher Scientific, Rockford, IL, USA).

2.10 Animal experiment

Male C57BL/6 mice which were around 8-week-old were obtained from Wuhan Shubeili Biotechnology Company. All animal experiments were performed under guideline standard procedures and approved by the Animal Ethics Committee of the Tongji Medical college, Huazhong University of Science and Technology. Mice were randomly divided into 4 groups and given with vehicle (0.1% DMSO in coin oil), 7-Mif (10 mg/kg weight or 20 mg/kg weight, dissolved in coin oil) once a day injected I.P. Then LPS (10 mg/kg) was inject intraperitoneally. 24 h later, mice were sacrificed and kidney, lung, and live were harvested.

2.11 Statistical analysis

The data are presented as mean ± S.D values. Student's t-test was performed for analyzing date from 2 different groups. p value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1 Effect of 7-Mif on HUVECs

To determine whether 7-Mif has cytotoxic effect on HUVECs, we used CCK8 kit to detected HUVECs' viability after treated with variable concentration of 7-Mif (1 μM, 5 μM, 10 μM, 50 μM and 100 μM). As demonstrated in Fig. 1B, 7-Mif show no cytotoxicity on HUVECs.

3.2 7-Mif attenuates endothelial inflammation in vitro

The expression of ICAM-1 and VCAM-1 significantly augmented in ECs treated with IL-1β. To figure out the effect of 7-Mif on inflammatory response of endothelial cells, we tested the expression of the ECs inflammation markers VCAM-1 and ICAM-1 after pretreatment with 7-Mif for 15min. Administration of 7-Mif at different concentrations (10 μM, 50 μM) mitigated the upregulated expression of VCAM-1 and ICAM-1 induced by IL-1β (Fig.2A). Consistently, the protein abundance of ICAM-1 and VCAM-1 were reduced significantly (Fig.2B). Then we utilized monocyte-EC adhesion assays by using THP-1 monocytes, the result showed less monocyte adhesion to the IL-1β treated HUVES (Fig.3). Hence, it proved that 7-Mif shows an inhibitory effect on endothelial inflammation in vitro.

3.3 7-Mif reduced the Luciferase Activity of the Promoters of ICAM-1 and VCAM-1

To further determine the mechanism of the inhibitory effect of 7-Mif through NF-κB signaling, we cloned reporter gene constructs into the Renilla plasmid and then transfected the 293T cells. As shown in Fig. 4, overexpression of p65 can significantly enhance the luciferase activity of both ICAM-1's and VCAM-1's promoters, while treatment with 7-Mif can reverse this effect.

3.4 7-Mif alleviates the NF-κB activation

The inflammatory response of endothelial cells is predominantly mediated by NF-κB[4]. As shown in Fig. 5, 7-Mif can decrease the phosphorylation of IκB and reverse its' degradation caused by IL-1β. Furthermore, the phosphorylation of p65 was reduced by 7-Mif. While the total of p65 kept unchanged. It suggests that the IL-1β inducing activation of NF-κB was suppressed by 7-Mif.

3.5 7-Mif restrains the Nuclear Localization of p65

The phosphorylation of IκB promotes its' degradation and hence allows the NF-κB to enter the nuclear. We used immunostaining to identify the effect of 7-Mif on NF-κB nuclear translocation. As shown in Fig. 6, 7-Mif could suppress the p65 nuclear translocation compared the HUVECS treated with IL-1β alone.

To confirm this find, we separated the nuclear protein from plasma protein and the IL-1β induced rising nuclear p65 was reversed by 7-Mif. On the contrary, the plasma p65 saw rise in HUVECs treated with 7-Mif.

3.6 7-Mif attenuates inflammatory response in vivo

In order to confirm the anti-inflammation ability of 7-Mif, we established model of the LPS-induced acute inflammation. 24 h after Intraperitoneal injection with 10 mg/kg LPS,histology showed a dramatic increasing neutrophil infiltration in lung. By pretreating 7-Mif (10 mg/kg weight or 20 mg/kg weight), the injuries caused by LPS was alleviated demonstrated by reducing neutrophil infiltration, interstitial edema and interstitial edema (Fig. 7A). In addition, we harvested the lung tissue and detected the expression of ICAM-1 and VCAM-1. The result implied that the expression of ICAM-1 and VCAM-1 in 7-Mif pretreated mice's lung was significantly decreased (Fig. 7B).

4. Discussion

Endothelial cells (ECs) are vital regulators of inflammatory processes. ECs can make the circulating immune cells' extravasation easier because of its' position between blood and tissue[15]. This makes ECs a potential therapeutic target for vascular disease. During inflammation, ECs response to multiple chemokines and cytokines, such as tumor necrosis factor (TNF)α and interleukin (IL)-1β[13].

After stimulated by IL-1β, the expression of adhesion molecules including ICAM-1 and VCAM-1 show up-regulated trend and then the plasm leukocytes are recruited. By this way, the ECs promote the progress of inflammation. Chronic vascular inflammation and ensuant monocytes' adhesion to the endothelium are critical at the initial stage for vascular diseases like atherosclerosis[9]. Here we demonstrated that a small molecular, 7-Mif, has the inhibitory effect of endothelial inflammation. 7-Mif can suppress the IL-1β-induced increasing expression of adhesion molecular in HUVECs both in mRNA levels and protein levels, furthermore 7-Mif reduces the THP1 cells adhesion to HUVECs. This result shows 7-Mif can suppress the endothelial inflammation and the potential of being a therapy for inflammatory vascular diseases.

NF-κB plays a critical role in the process of inflammation by mediating not only the expression of pro-inflammatory cytokines, but also the expression of adhesion molecules and chemokines[14]. Cytokine upregulation of matrix metalloproteinase (MMP)-1, -3 and − 9 produced by vascular smooth muscle cells also need the enrollment of NF-κB and its' inactivation can maintain plaque stabilization[3]. In this study, we revealed the mechanism by which 7-Mif can inhibit the inflammation of endothelium through the inactivation of NF-κB pathway. The NF-κB transcription factor consist of homo- or hetero-dimers of RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), or p52/p100 (NF-κB2), and p65/p50 heterodimers are the main activator of transcription in endothelial cells[10]. In stable cells, NF-κB is captured in the cytoplasm by interaction with Inhibitor of κB (IκB)[6]. Being excited by the in inflammatory signaling, IκB kinase (IKK) complex will phosphorylate IκB lead to its' degradation[2]. Thus, NF-κB is freed and translocated into the nucleus and regulate gene expression[17]. Our date showed that 7-Mif can lighten the phosphorylation of IκBα and its' degradation, as well as the phosphorylation of p65. Then we utilized immunostaining to

determine the location of p65 in HUVECs. After administrated with IL-1β, p65, mainly resisting in cytosol under basic condition, translocates in the nuclear. By pretreated with 7-Mif, p65 was restrained in cytosol in IL-1β-stimulated HUVECs. What's more, we separated nuclear and cytoplasmic protein of HUVECs, and the result of Western blotting shows nuclear p65 reduced by pretreatment of 7-Mif, and the accumulation of p65 in cytosol is higher than the group only treated with IL-1β. Taken together, we illustrate the protective effect of 7-Mif against inflammation in endothelium and the effect was through inhibiting the NF-κB signaling.

Additionally, endothelial cells play a crucial role in the LPS-induced inflammation[5]. In response to acute inflammation, ECs will secret pro-inflammatory cytokines, which further activates monocytes, neutrophils and endothelial cells, eventually causing endothelial dysfunction characterized by increasing vascular permeability. We establish acute inflammation animal models by administrating LPS for 24 h. The results show that mice from group that pretreated with 7-Mif had less plasma leakage and leukocytes recruitment. Our finding suggests that 7-Mif has positive effect on vascular inflammatory response and more exploration into the possible uses of it, such as in atherosclerosis, are needed.

In a nutshell, our study reveals that the inhibitory effect of 7-Mif on endothelial inflammation both in vivo and in vitro. And it is through suppressing the NF-κB signaling activation that 7-Mif act as an anti-inflammation mediator. The ability of attenuating inflammatory gene expression, reducing monocytes recruitment and vascular permeability of 7-Mif makes it a potential candidate for treating vascular diseases.

Declarations

Funding

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Conflicts of interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

M. Liang and K. Huang conceived of and designed the experiments. X. Zhu and C. Wang performed the experiments and prepared the manuscript. M. Liang and K. Huang prepared and revised the manuscript. All authors gave final approval.

Ethics approval

All animal experiments were performed under guideline standard procedures and approved by the Animal Ethics Committee of the Tongji Medical college, Huazhong University of Science and Technology.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

Not applicable.

Code availability

Not applicable.

Acknowledgements

Not applicable.

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Figures

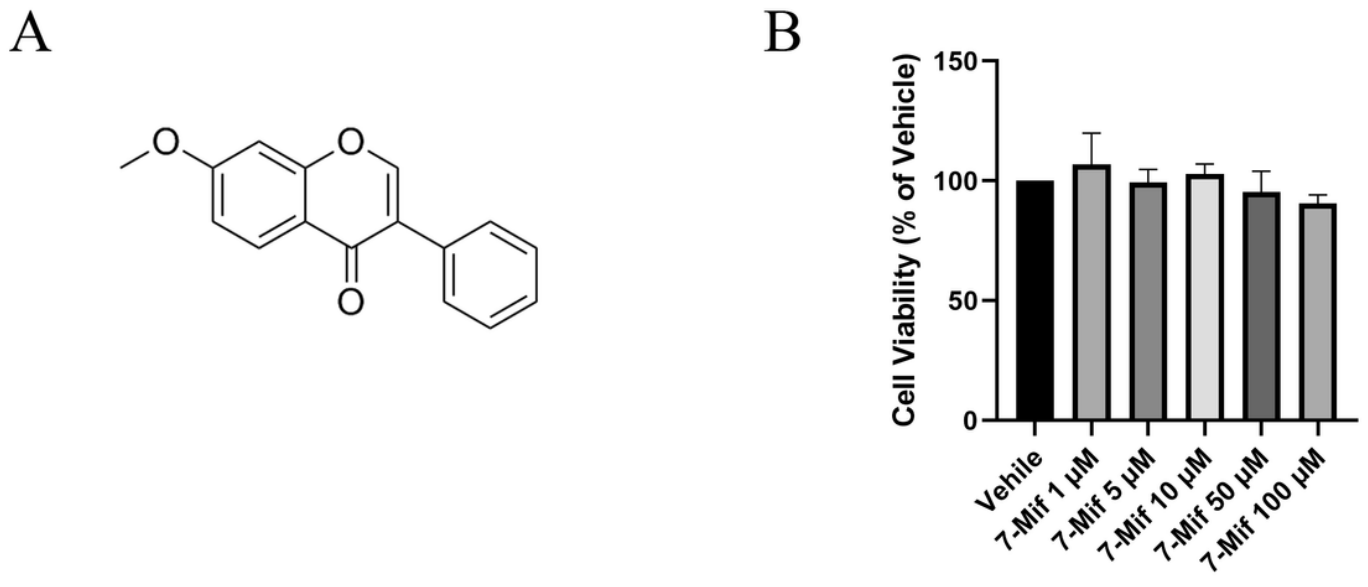


Figure 1

(A) Chemical structure of 7-Mif. (B) Effect of 7-Mif on HUVECs. 7-Mif has no cytotoxic effect at the maximum concentration of 100 μM on HUVECs.

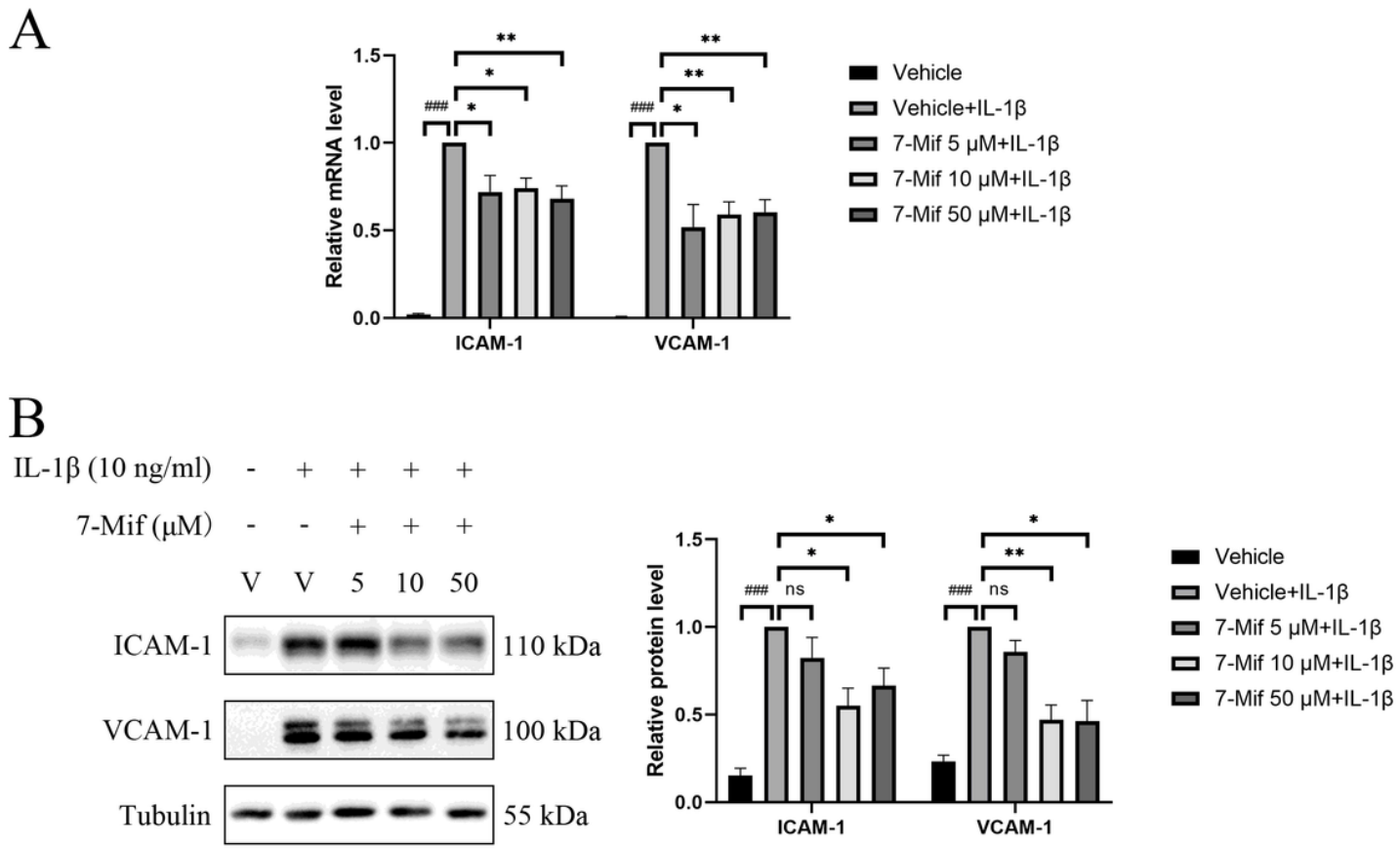
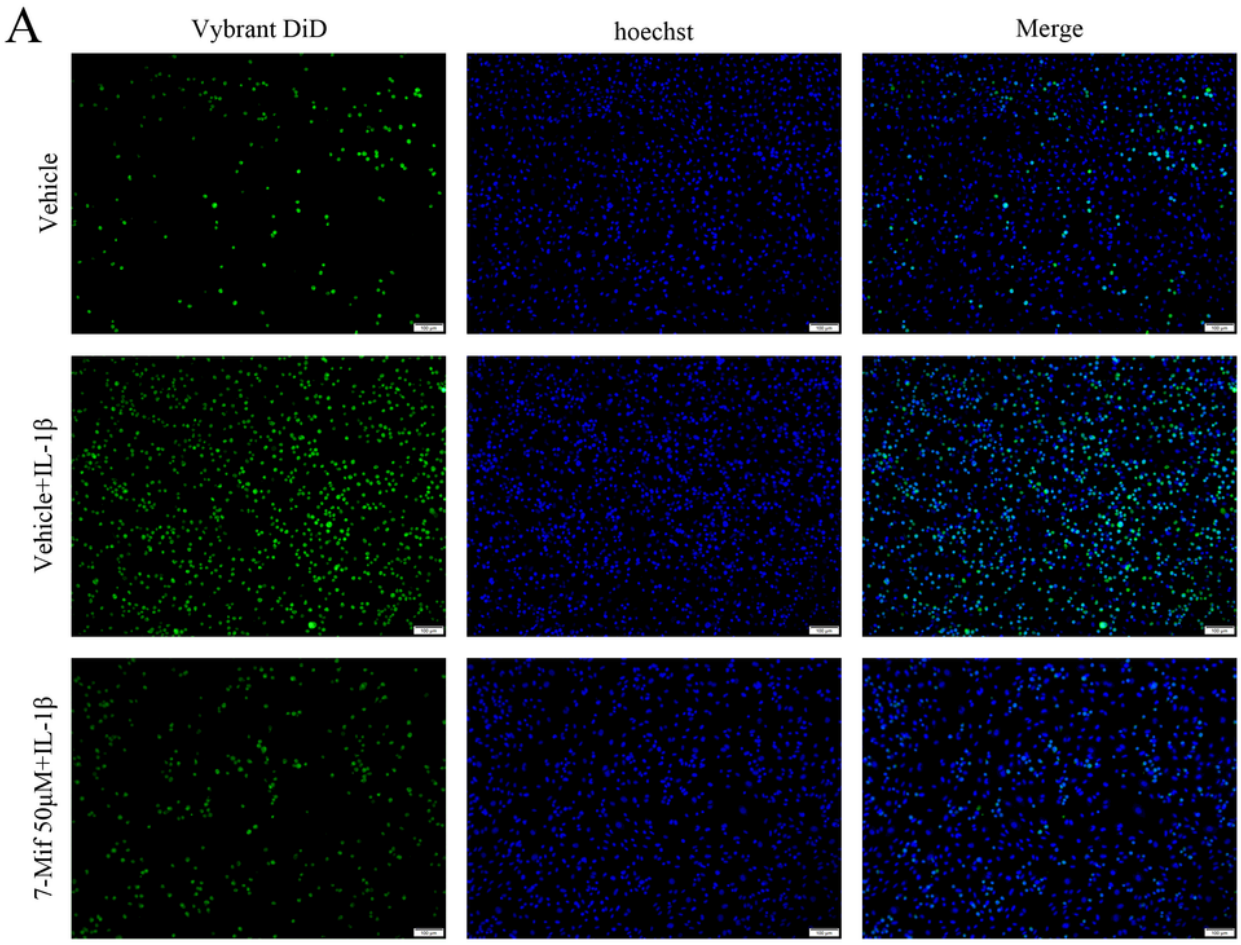


Figure 2

7-Mif attenuates endothelial inflammation in vitro. HUVECs were incubated with various concentrations of 7-Mif for 15 min, and subsequently treated with IL-1β (10 ng/mL) for 4 h. (A) mRNA expression levels of ICAM-1 and VCAM-1 of were identified by RT-PCR (n=3). (B) Representative images and quantification for protein abundance of ICAM-1 and VCAM-1 were identified by western blotting (n=3). ### p<0.001 versus vehicle group, * p<0.05, ** p<0.01 versus vehicle+IL-1β group.



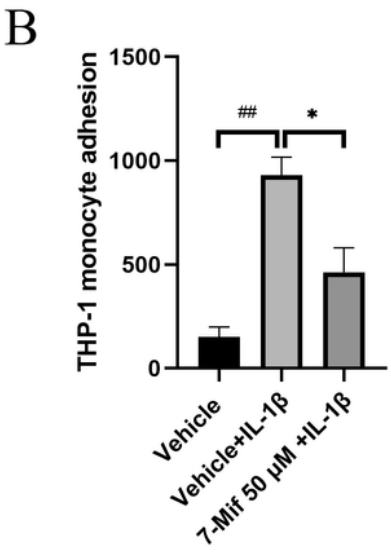


Figure 3

7-Mif attenuates endothelial inflammation in vitro. (A) Representative images of THP-1cells-HUVECs adhesion are shown (scale bar represents 100 μm). HUVECs' nuclei were presented in blue (Hoechst), and THP-1 was presented in green (Vybrant DiD). The amount of binding THP-1 cells are presented as Green fluorescence. (B) Quantification of the percentage of adherent THP-1 cells. n=3 ##p<0.01 versus vehicle group, *p <0.05 versus vehicle+IL-1β group.

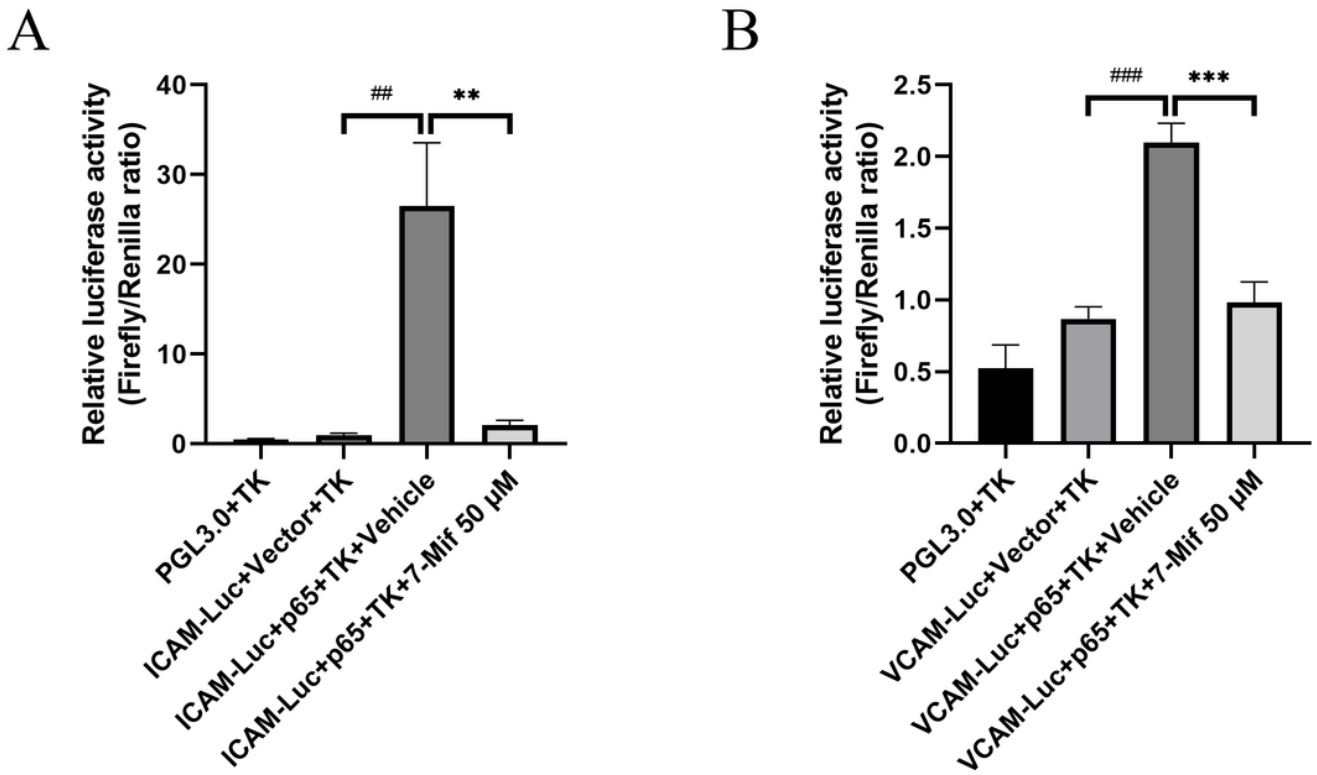


Figure 4

7-Mif reduced the Luciferase Activity of the Promoters of ICAM-1 and VCAM-1. (A) The luciferase activity of ICAM-1's promoter is shown (n=3). ##p <0.01 versus ICAM-Luc+Vector+TK group, **p<0.001 versus ICAM-Luc +p65+TK+Vehicle group. (B) The luciferase activity of VCAM-1's promoter is shown (n=3). ###p <0.001 versus VCAM-Luc+Vector+TK group, ***p <0.001 versus VCAM-Luc+p65+TK+vehicle group. Luciferase activity is represented as fold change versus empty vector.

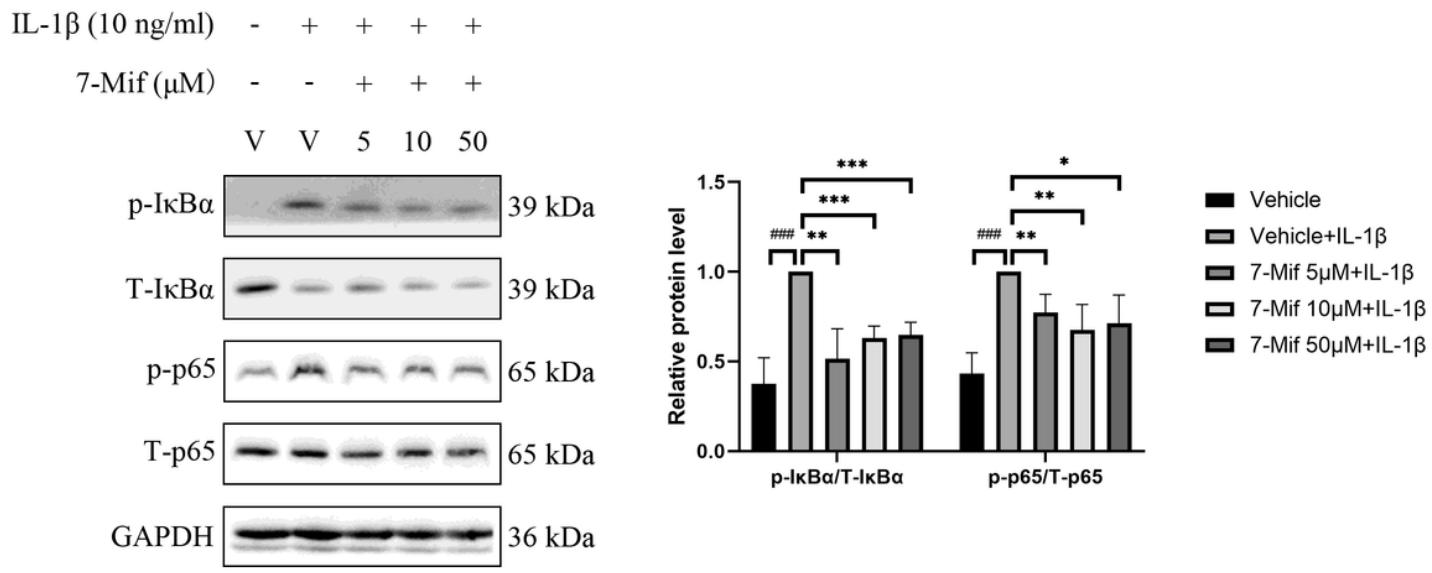


Figure 5

7-Mif alleviates the NF-κB activation. HUVECs were treated with 7-Mif (5 μM, 10 μM, or 50 μM) for 15 min and subsequently stimulated with 10 ng/ml IL-1β for 4 h. Representative images and quantification for p65 and IκBα protein phosphorylation levels are shown. (n=3) ### p<0.001 versus vehicle group, * p <0.05, ** p <0.01, ***<0.001, versus vehicle+IL-1β group.

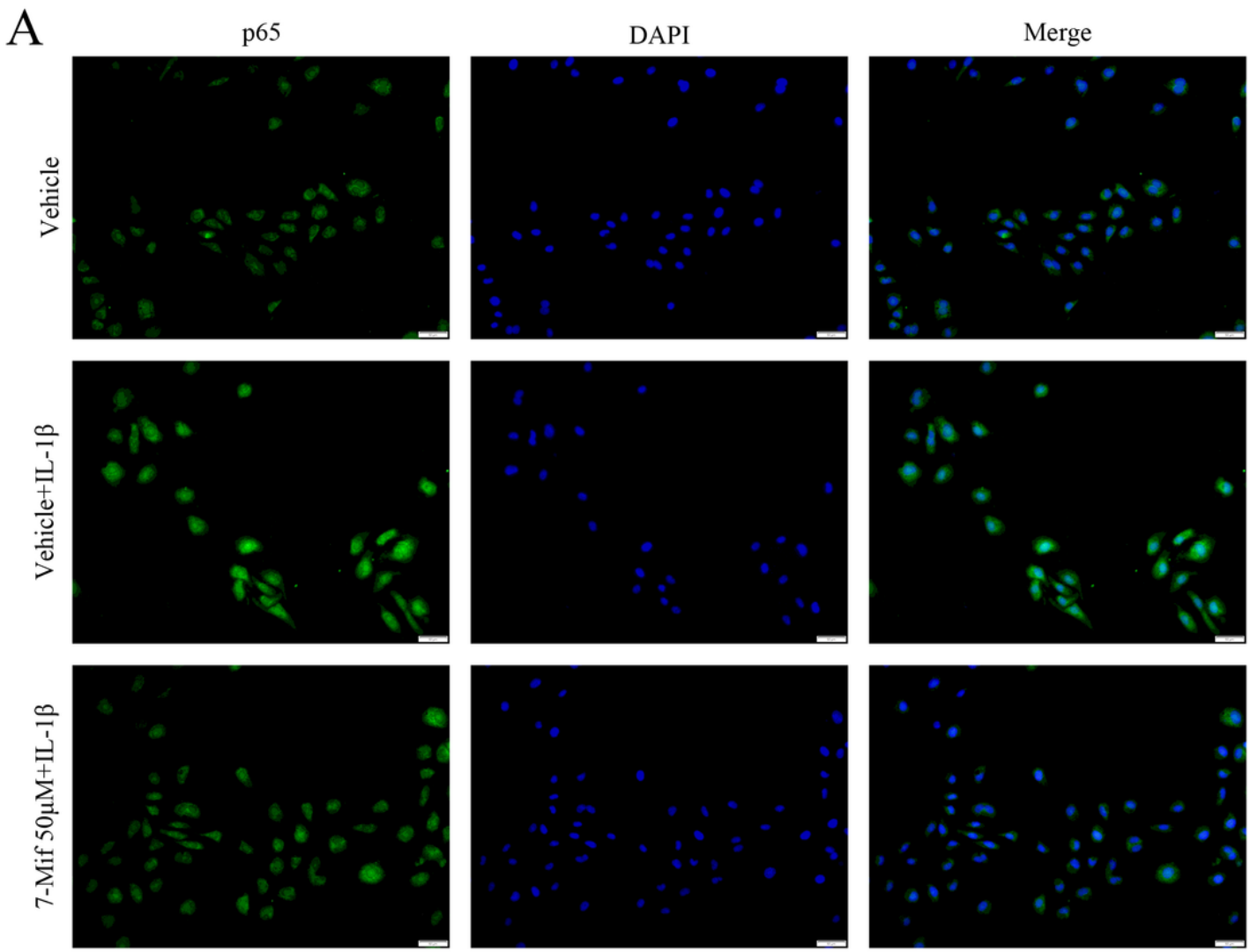


Figure 6

(A) Representative images of p65 translocation by immunofluorescence in IL-1β-stimulated HUVECs are shown (scale bar represents 50μm). Nuclei were presented in blue (DAPI), and p65 was presented in green (Alexa Fluor 488). Localization of p65 was presented by green fluorescence (n=3). (B) p65 protein abundance in the cytoplasm (left) and nucleus (right) of IL-1β-stimulated HUVECs was measured by western blot (n=3).

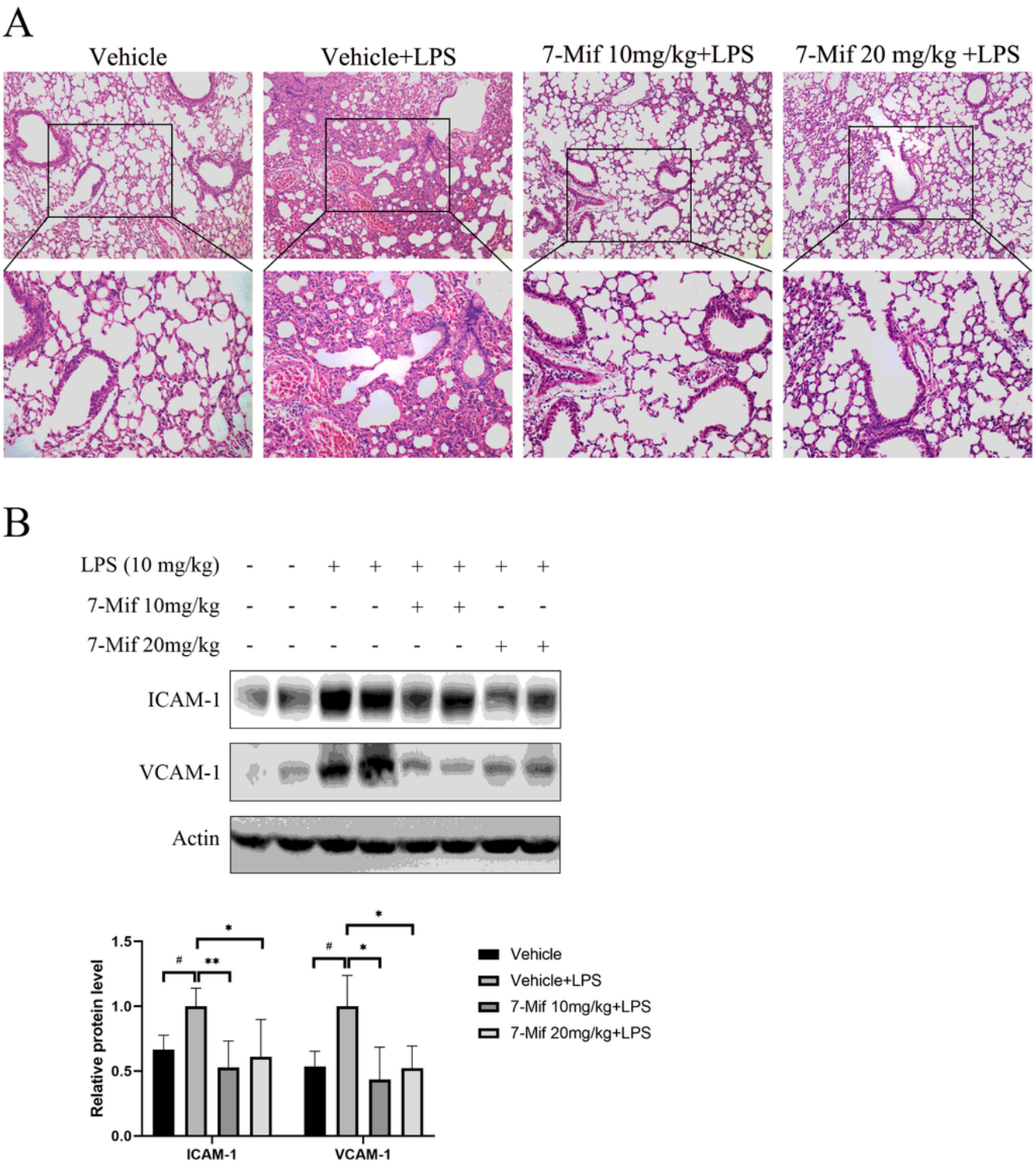


Figure 7

7-Mif attenuates inflammatory response in vivo. (A) Representative images present H&E staining for accumulation of inflammatory cell in the lung. (B) Representative images and quantification of ICAM-1 and VCAM-1 protein abundance in the lung tissue were identified by western blotting. # $p<0.001$ versus vehicle group, * $p<0.05$, ** $p<0.01$, versus vehicle+LPS group (n=3).